Neuroendocrine Regulation of Hypothalamo-Pituitary Function in the Fetus

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Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has beeen previously accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

During fetal development there is a progressive maturation of the hypothalamo-pituitary-adrenal (HPA) axis which is responsible for co-ordinated fetal maturation and the onset of birth. The aim of this thesis is to investigate the neuroendocrine regulation of the developing HPA axis. The first approach was to investigate the role of putative neurotrophic factors in the functional maturation of hypothalamic arginine vasopressin (AVP) neurons. The role of AVP in the release of adrenocorticotropic hormone (ACTH) has been extensively demonstrated but little is known about the factors controlling the development of AVP neurons. The objective of this study was to chart the growth and development of AVP neurons in cell culture in response to various neurotrophic factors. Functional maturation of the neurons was assessed in terms of the secretory response to potassium-induced depolarisation whilst development of neuronal morphology was to be monitored by immunocytochemistry. Fetal rat hypothalamic neurons were cultured in the presence and absence of insulin-like growth factor-1 (IGF-1), which has been implicated as a putative neurotrophic factor, for periods of up to 20 days. IGF-1 had no significant effect on either basal or stimulated levels of AVP secretion, suggesting that AVP neurons do not appear to be the specific target population for IGF-1 within the developing fetal hypothalamus. Despite extensive screening of several different antibodies, we were unable to specifically identify AVP neurons in culture by immunocytochemistry.

The second approach to studying the neuroendocrine regulation of the HPA axis utilised the sheep as an animal model as it affords chronic in utero manipulation of the fetus. In the ovine fetus, maturation of the HPA axis is pivotal in the onset of parturition. The hypothesis that POMC-derived peptides of the ovine fetal pituitary are subject to tonic dopamine inhibition throughout gestation was investigated. Removal of this inhibitory tone may induce synthesis and release of adrenal active peptides which in turn elicit cortisol secretion from the fetal adrenal gland. The approach to the hypothesis was to investigate the effects of dopaminergic manipulation of the fetus, on the secretion of POMC-derived peptides and cortisol into the fetal circulation, on pituitary content of POMC-derived peptides and on the level of POMC gene expression. Specifically, fetal sheep at day 131 gestation (gestation=145 days) were treated with a 72 hour intravenous administration of the dopamine agonist bromocriptine, the dopamine antagonist sulpiride or vehicle control. Treatment with bromocriptine significantly decreased the concentration of α-MSH in the fetal plasma,

represented by an abolition of the pulsatile mode of secretion of α-MSH. In response to sulpiride infusion, plasma concentrations of both α-MSH and ACTH were significantly elevated, with α-MSH secretion displaying increased pulse amplitude with decreased pulse frequency. By contrast, the pulsatile characteristics of ACTH were unaltered by sulpiride infusion. Despite increased plasma concentrations of α-MSH and ACTH following sulpiride infusion, the concentration of cortisol in the fetal plasma was unaltered.

α-MSH and ACTH were identified by immunohistochemistry and determination of peptide content in the pars distalis and pars intermedia of the fetal pituitary. The content of ACTH in the pars intermedia tended to increase in response to bromocriptine and decrease in response to sulpiride treatment. Pituitary α-MSH content was unaffected by either treatment. POMC mRNA levels in the fetal pars intermedia was significantly reduced following bromocriptine treatment and were unaltered by treatment with sulpiride. The level of POMC gene expression in the pars distalis was not significantly altered by either treatment.

Studies on the ontogeny of POMC gene expression and the development of α-MSH and ACTH immunoreactivity revealed that POMC gene expression, ACTH and α-MSH immunoreactivity can be detected as early as day 40 gestation. ACTH immunoreactivity was detected in both the pars intermedia and pars distalis of the pituitary. In contrast, α-MSH immunoreactivity was confined to the pars intermedia at most gestational ages examined. However, at day 40-50 gestation α-MSH immunopositive cells were also identified in the pars distalis. POMC mRNA levels were consistently higher in the pars intermedia compared to the pars distalis and increased progressively from day 40 until day 134 gestation. In the pars distalis, POMC mRNA levels were high at day 40 gestation, decreased at day 50 and then rose progressively until day 134 gestation.

Fetal plasma prolactin concentrations were also influenced by treatment with bromocriptine and sulpiride. Secretion of prolactin into the fetal plasma was influenced by season, with concentrations peaking in the summer months and decreasing to very low levels in the winter. Winter prolactin concentrations could be further suppressed by infusion of bromocriptine. Conversely, sulpiride treatment significantly increased prolactin concentrations in fetuses treated in the winter months

however, concentrations in sulpiride-treated winter fetuses were still lower than those of vehicle controls in the summer months. The seasonal alteration in plasma prolactin concentrations was mirrored by a significant decline in the number of immunopositive lactotrophs in the winter. The number of immunopositive lactotrophs was unaffected by bromocriptine or sulpiride infusion in either season.

These results demonstrate the existence of an endogenous inhibitory system regulating the release of ACTH, α-MSH and prolactin from the fetal pituitary gland. However, these results do not support the hypothesis that peptides released upon removal of the inhibitory dopaminergic tone are capable of eliciting the secretion of cortisol from the fetal adrenal gland.

Abbreviations

α-MSH	α-melanocyte stimulating hormone
ACTH	Adrenocorticotropic hormone
AL	Anterior lobe
AVP	Arginine vasopressin
AVP-NP	AVP associated neurophysin
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid binding globulin
CLIP	Corticotropin-like intermediate peptide
CRH	Corticotropin releasing hormone
CTP	Cytosine triphosphate
DAB	Diaminobenzidine
DARS	Donkey anti-rabbit serum
DASS	Donkey anti-sheep serum
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate
HPA	Hypothalamo-pituitary-adrenal
HPD	Hypothalamo-pituitary disconnection
HRP	Horse radish peroxidase
IGF-1	Insulin-like growth factor-1
IL	Intermediate lobe
ir-	immunoreactive
IRMA	Immunoradiometric assay
kb	Kilobases
mRNA	messenger RNA
NBT	Nitroblue tetrazolium
NIL	Neurointermediate lobe
NL	Neural lobe
NRS	Normal rabbit serum
NSE	Neuron specific enolase
NSS	Normal sheep serum

PRF	Prolactin-releasing factor
PBS	Phosphate buffered saline
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
RNase	Ribonuclease
RNA	Ribonucleic acid
SARB	Swine anti-rabbit biotinylated
SDS	Sodium dodecyl sulphate
SFCM	Serum-free culture medium
TBE	Tris buffered EDTA
TBS	Tris buffered saline
TEA	Triethanolamine
TRIS	Tris(hydroxymethyl)aminomethane
UTP	Uridine triphosphate

Chapter 1. Introduction

Premature birth is a major problem in clinical obstetrics with 5-9% of all births in the United Kingdom occurring preterm. Preterm births are the leading cause of perinatal mortality and morbidity, accounting for almost 85% of cases of perinatal death which are not attributed to congenital malformations. Moreover, surviving preterm infants have an increased risk for long-term morbidity, including cerebral palsy and mental retardation. Whilst about one-third of these premature deliveries occur in association with infection, for the majority of presentations, the mechanisms initiating preterm labour remain undetermined. Indeed, little is known about the mechanisms which govern the precise timing of the onset of normal labour or whether the same mechanisms are involved in the onset of preterm birth.

The sequential maturation of the hypothalamo-pituitary-adrenal (HPA) axis during fetal life plays a significant role in prenatal development, preparing the fetus for birth and subsequent existence in the external environment. Corticotrophin-releasing factors, arginine vasopressin (AVP) and corticotrophin releasing hormone (CRH) are secreted from the fetal hypothalamus and act at the pituitary gland to elicit the release of adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the secretion of cortisol from the fetal adrenal gland. Increased cortisol secretion in late gestation is essential for maturation of the fetal lung and gut and this knowledge is now exploited clinically by the use of corticosteroids to advance lung maturation in cases of threatened preterm delivery. In addition, increased fetal cortisol levels induce biochemical changes in the steroid biosynthesis pathway of the placenta resulting in an increase in oestrogen and a decline in progesterone production. This shift in the oestrogen to progesterone ratio ultimately stimulates enhanced contractility in the myometrium and culminates in the initiation of labour. Thus, the maturation of the fetal HPA axis is pivotal in the preparation for life outwith the uterus and for the initiation of parturition.

This thesis aims to investigate the regulation of the hypothalamo-pituitary axis in the late gestation fetus. Whilst the role of the hypothalamic releasing factors CRH and AVP in the release of ACTH from the fetal pituitary gland has been well demonstrated, little is known about the factors influencing the development of these neuropeptide neurons within the fetal hypothalamus. The study presented in Chapter 4 was designed to investigate the role of putative neurotrophic factors in the growth and functional maturation of hypothalamic AVP neurons. However, it

became clear that the culture system was inappropriate for the needs of the study as it was not possible to specifically identify AVP neurons within the culture system.

The remainder of the studies presented in this thesis concentrate on the ontogeny and neuroendocrine regulation of the pituitary component of the HPA axis, and focused primarily on the proopiomelanocortin (POMC) family, of which ACTH is a member. These studies utilised the fetal sheep as an animal model at it affords chronic in utero manipulation of the fetus. In addition, the pivotal role of the fetal HPA axis in the initiation of parturition in the sheep has been widely demonstrated. However, although ACTH is believed to be the main regulating hormone for fetal adrenal cortisol during the last third of gestation, adrenal activation in the absence of increased plasma ACTH concentrations and discordance in the pulse characteristics of the ACTH and cortisol has led to the suggestion that ACTH may not be solely responsible for driving cortisol secretion from the fetal adrenal gland in late gestation. In this respect, α-MSH, a peptide secreted from the intermediate lobe of the fetal pituitary has been implicated in the induction of cortisol secretion from the adrenal gland. As the secretion of α-MSH from the adult pituitary gland has been shown to be under the inhibitory influence of dopamine, the studies presented in Chapters 6-8 of this thesis were designed to test the hypothesis that POMC-derived peptides of the fetal pituitary gland are subject to tonic dopaminergic regulation during fetal life and that removal of this inhibitory tone will result in the release of adrenal active peptides which elicit cortisol secretion from the fetal adrenal gland. Specifically, the role of dopamine in the regulation of POMC-derived peptide secretion, on pituitary content of these peptides, and on the level of POMC gene expression was investigated.

As it has been demonstrated that plasma prolactin concentrations in the ovine fetus are modulated both by dopamine and external photoperiod, an additional study to determine the extent to which season influences the dopaminergic regulation of prolactin in the late gestation fetus is presented in Chapter 9.

Chapter 2. Review of the literature

2.1 The fetal pituitary-adrenal axis and fetal maturation

The significance of the fetal pituitary-adrenal axis in the timing of parturition was first realised in the 1960's when sheep grazing on *Veratrum californicum* were seen to deliver deformed fetuses at the end of an extended gestational period. Further investigation of the fetuses revealed lesions of the fetal brain and pituitary and hypoplasia of the adrenal gland (Binns, Anderson and Sullivan, 1960). This led to the suggestion that disruption of the fetal pituitary-adrenal axis was responsible for anomalous fetal maturation and the failure to deliver at term.

Liggins and co-workers did a series of elegant experiments to investigate the extent to which the fetal pituitary-adrenal axis was involved in parturition. Electrocoagulation of the fetal pituitary resulted in delayed parturition in those fetuses in which more than 70% of the pituitary had been destroyed (Liggins, Kennedy and Holm, 1967). This experiment was followed by one in which the major pituitary hormones were replaced either individually or in combination in an attempt to identify the specific factors from the pituitary that were necessary to precipitate delivery. Administration of growth hormone, prolactin, luteinizing hormone or follicle-stimulating hormone alone had no effect on the timing of parturition. However, administration of these hormones concomitantly with adrenocorticotropic hormone (ACTH) or administration of ACTH alone resulted in the onset of parturition. Moreover, administration of ACTH with a corticosteroid synthesis inhibitor, metyrapone prevented the induction of parturition (Liggins, 1968). Liggins (1969) went on to show that intrafetal infusion of cortisol was capable of inducing premature delivery in the sheep. Moreover, fetuses delivered prematurely following activation of the fetal adrenal were viable, implicating a role for glucocorticoids in fetal lung and organ maturation. The timing of parturition is unaltered by maternal hypophysectomy or maternal administration of ACTH or glucocorticoids (Liggins, Fairclough, Grieves, Kendall and Knox, 1973) illustrating the active role of the fetus in this regard.

The demonstration that fetal plasma cortisol concentrations rise dramatically during the final days before parturition (Basset and Thornburn, 1968) gave credence to the now generally accepted hypothesis that activation of the fetal hypothalamo-pituitary-adrenal axis gives rise to parturition in the sheep.

2.2. Ontogeny of the fetal hypothalamic-pituitary-adrenal (HPA) axis

2.2.1. Plasma concentrations of ACTH and cortisol

During fetal development there is a progressive increase in plasma concentrations of ACTH and cortisol in the last 15-20 days of gestation and a dramatic pre-parturient surge in both hormones in the final few days before birth.

Early studies (Rees, Jack, Thomas and Nathanielsz, 1975) suggested that the increase in plasma ACTH concentrations follow the cortisol surge. However, with more rigorous sampling protocols and sensitive radioimmunoassays the rise in ACTH has been shown to precede the increase in cortisol concentrations (Norman, Lye, Wlodek and Challis, 1985, Challis and Brooks, 1989). Brooks and Challis (1991) demonstrated that the final preparturient surge in ACTH and cortisol concentrations is highly pulsatile, with cortisol pulses displaying increased pulse amplitude and decreased pulse frequency 1-2 days compared to 3-4 days prior to parturition. ACTH pulses occurred on average once every 40 minutes but there was no change in the pulse frequency or amplitude in the 4 days before birth. Interestingly, in this study and one other (Apostolakis, Longo, Veldhuis and Yellon, 1992), not all ACTH pulses were coincident with cortisol pulses suggesting that ACTH is not solely responsible for driving cortisol secretion from the fetal adrenal in late gestation and that other factors may be involved in regulation of fetal adrenal gland secretion.

The fetal sheep pituitary gland contains several high molecular weight species that are immunoreactive for ACTH (Silman, Holland, Chard, Lowry, Hope, Thomas and Nathanielsz, 1979; Jones and Roebuck, 1980). These high molecular weight molecules are present in greater amounts in the fetus than in the adult and the late gestation increase in adrenal activity coincides with a marked increase in the relative

proportion of $ACTH_{1-39}$ in the circulating fetal plasma compared to the larger molecular weight peptides (Roebuck, Jones, Holland and Silman, 1980). Jones and Roebuck (1980) cultured adrenal cells with three high molecular weight ACTH molecules (50-60 kD, 30kD and 20kD) in the presence or absence of $ACTH_{1-39}$ and demonstrated an inhibition of ACTH-induced cortisol production, suggesting that the high molecular weight molecules act as antagonists to ACTH. If this is the case, a change in the ratio of the high molecular weight molecules and $ACTH_{1-39}$ in the fetal plasma could influence the late gestation surge from the adrenal gland. Brieu and Durand (1987) used an *in vitro* bioassay to compare the biological and immunological properties of ACTH-like activity released from cultured pituitary cells between day 63 and 144 gestation and newborn lambs. During fetal life, levels of immunoreactive ACTH were always higher than bioactive ACTH. However, the ratio of bioactive to immunoreactive ACTH increased with advancing gestation implying an increase in drive to the adrenal by bioactive ACTH.

Recently, the secretion of $ACTH_{1-39}$ in late gestation relative to its precursors, proopiomelanocortin (POMC) and pro-ACTH has been investigated using perifused slice cultures (McMillen, Merci, White, Crosby and Schwartz, 1995). Slices of anterior pituitary from three gestational ages, day 106-113, day 120-136 and day 140-143 were analysed and a significant increase in the secretory rate of $ACTH_{1-39}$ reported between day 120-136 and day 140-143 gestation. In contrast, there was no change in the rate of secretion of the precursors resulting in an increase in the ratio of $ACTH_{1-39}$: ACTH precursors between 120 and 143 days of gestation.

This alteration in the post-translational processing of POMC late in gestation to favour production of $ACTH_{1-39}$ may be associated with the change in corticotroph cell type seen at this time (section 2.2.3) and may be important in the increase in trophic drive to the adrenal prior to parturition.

2.2.2. Corticotrophin releasing factors of the fetal hypothalamus

Following the discovery in 1981 (Vale *et al* .) of a 41-amino acid residue in the ovine hypothalamus that acted as a corticotrophin releasing factor, now referred to as corticotrophin releasing hormone (CRH), and the subsequent confirmation of arginine vasopressin (AVP) as an ACTH-releasing factor (Antoni, 1986; 1993, for

review) rapid advances have been made in the study of these hypothalamic releasing factors in the control of the ACTH- adrenal axis.

CRH and AVP neurons from the parvocellular portion of the hypothalamic paraventricular nucleus (PVN) project extensively to the external lamina of the median eminence (Swanson, Sawchenco, Rivier and Vale, 1983) where AVP and CRH are released into the portal blood supply. In addition to the presence of hypothalamic neuropeptides in the portal blood, high concentrations of catecholamines, dopamine, norepinephrine, and epinephrine are also secreted into the portal circulation (Paradisi, Frank, Grossi, Venturoli, Porci, Capelli, Galassi and Flamignin, 1989). Development of the portal connection between the hypothalamus and pituitary in the fetal sheep is complete by day 45 gestation (Matwijiw, Thliveris and Fairman, 1989, Levidiotis, Wintour, McKinley and Oldfield, 1989). AVP positive terminals have been identified in the external lamina of the median eminence as early as day 42 of gestation in the fetal sheep (Levidiotis, Oldfield and Wintour, 1987). Although CRH positive connections to the median eminence were not identified until day 105 gestation in this study, CRH neurons were detected in the paraventricular nucleus from day 90 gestation. More recently, CRH containing cell bodies have been identified in the hypothalamus by day 49 gestation with corresponding positive fibres projecting to the median eminence (Watabe, Levidiotis, Oldfield and Wintour, 1991). The coexistence of CRH and AVP within neurosecretory vesicles in the rat median eminence has been demonstrated with double-labelling immunocytochemistry (Whitnall, Mezey and Gainer, 1985) with 50% of the CRH containing neurons also containing AVP (Whitnall, Smyth and Gainer, 1987). These workers have also show that AVP-containing and AVP-deficient CRH neurons have differential distributions within the PVN (Whitnall and Gainer, 1988) and that the AVP-containing population of CRH neurons are selectively activated in response to stress (Whitnall, 1989).

Thus, the neuronal connections from the PVN to the median eminence and the portal blood supply connecting the hypothalamus with the fetal pituitary are already intact in the first third of gestation.

Contents of immunoreactive CRH and AVP in the fetal sheep hypothalamus increase with advancing gestational age (Currie and Brooks, 1992), with the greatest

increases between day 100 to day 130 gestation. AVP is always found in greater concentrations than CRH. The ratio of AVP to CRH in the hypothalamus falls with advancing gestational age (Brieu, Tonon, Lutz-Bucher and Durand, 1989, Currie and Brooks, 1992) reflecting a dramatic increase in the concentration of CRH, thought to play a more significant role in late gestation (Norman *et al.*, 1985). CRH release from perifused hypothalamic pieces also increases with advancing gestational age (Brooks, Power, Jones, Yang and Challis, 1989). This rise in immunoreactive CRH released from the mediobasal hypothalamus is in accordance with significantly increased levels of CRH mRNA seen in the paraventricular nucleus of the intact fetus between days 105-107 and days 128-130 gestation (Myers, Myers, Grober, Nathanielsz, 1993). The increase in CRH mRNA and CRH secretory activity precedes the surge of adrenocortical activity seen in the last days of gestation. More recently, Keiger *et al.* (1994) have extended these observations demonstrating that the abundance of CRH mRNA continues to increase with a two-fold increase in CRH mRNA in day 140-142 gestation hypothalami compared to day 128-138 gestation.

2.2.3. Development of the fetal pituitary gland

The pituitary gland contains three distinct regions: the anterior lobe (pars distalis), the intermediate lobe (pars intermedia) and the neural lobe (pars nervosa). The intermediate and anterior lobes of the pituitary constitute the true endocrine gland whereas the neural lobe is not in itself an endocrine organ but rather it consists of a bundle of nerve endings with cell bodies originating in the hypothalamus. A common epithelial progenitor gives rise to five distinct cell types which produce the anterior pituitary hormones: sommatotrophs produce growth hormone, lactotrophs produce prolactin, thyrotrophs produce thyroid-stimulating hormone, gonadotrophs produce follicle-stimulating and luteinizing hormone and corticotrophs produce ACTH. The appearance of these five glandular epithelial cell types is both spatially and temporally regulated such that each cell type arises at a different period in gestation and occupies a specific area within the gland. Of these hormones ACTH produced by fetal corticotrophs is significant in the development of the HPA axis.

Fetal pituitary corticotrophs have been identified as early as day 40 gestation (Perry, Mulvogue, McMillen and Robinson, 1985). By day 87 gestation, they exist as two morphologically distinct types (Perry *et al.*, 1985). Fetal type corticotrophs are

columnar, palisade cells that stain weakly for ACTH while adult type corticotrophs are small angular cells that stain more intensely for ACTH. Fetal corticotrophs are the dominant type in the anterior pituitary until day 130 gestation, after which time the numbers decline and more adult type cells are present (Mulvogue, McMillen, Robinson and Perry, 1986). It is possible that the switch from fetal to adult type corticotrophs represents a shift in responsiveness to hypothalamic factors such that the adult corticotrophs are more responsive to CRH. This would correlate with the observed increase in stimulatory capacity of CRH and corresponding decrease in AVP stimulation late in gestation (Norman *et al.*, 1985; Norman and Challis, 1987a). The same workers also describe the presence of an intermediate-type cell (Antolovich Perry, Trahair, Silver and Robinson, 1989) with characteristics of both adult and fetal type cells suggesting that the adult population arises from the fetal population following a transitional period. The relative proportions of fetal to adult type corticotrophs can be influenced by bilateral adrenalectomy at day 120 gestation or by premature treatment of fetuses with exogenous cortisol between day 109 and day 115 gestation (Antolovich *et al.*, 1989) implicating cortisol from the fetal adrenal gland in maturation of corticotrophs from fetal to adult type and illustrating the feedback effect of the adrenal gland on the fetal pituitary. Destruction of the paraventricular nucleus (McDonald, Hoffman and Nathanielsz, 1992) and fetal hypothalamo-pituitary disconnection (HPD : Antolovich, McMillen, Robinson, Silver, Young and Perry, 1991) also prevents normal maturation of the fetal corticotrophs in the anterior pituitary demonstrating the need for an intact hypothalamo-pituitary connection. It is possible that the influence of HPD and PVN lesion on corticotroph maturation reflects altered cortisol concentrations after these procedures. However, whilst PVN lesioned fetuses fail to show increased cortisol concentrations with advancing gestation (McDonald and Nathanielsz, 1991), Antolovich *et al.* (1991) reported that cortisol concentrations in HPD fetuses were not significantly different from those in sham-operated control fetuses.

The intermediate lobe of the pituitary is a compact region of glandular epithelium that develops between the anterior and neural lobes. Melanotrophs of the intermediate pituitary secrete α-melanocyte stimulating hormone (α-MSH), corticotrophin-like intermediate peptide (CLIP) and acetylated β-endorphin. Cytoplasmic differentiation of the pars intermedia cells is completed by day 100 gestation. High levels of exocytosis and the presence of fenestrated capillaries, both

accepted morphologies of peptide secreting organs, have been demonstrated (Perry, Robinson and Ryan, 1982) although the intermediate lobe is less well vascularised than the anterior lobe. The same authors found a greater frequency of exocytosis in the intermediate lobe cells from late gestation fetal sheep compared to those of the adult intermediate lobe, suggesting that the peptides secreted by the intermediate lobe may have an important role in the fetus. Both stimulatory and inhibitory nerve terminals have been identified terminating directly on the epithelial cells of the intermediate lobe. Stimulatory β-adrenergic (Tilders, Post, Jackson, Lowry, and Smelik, 1981) and serotonergic (Westlund and Childs, 1982) fibres have been identified in the intermediate lobe of the rat and GABAergic (Vincent, Hokfelt and Wu, 1982) and dopaminergic (Tilders and Smelik, 1978) fibres terminating in the intermediate lobe have been implicated in the inhibition of α-MSH release from melanotrophs. The intermediate lobe is prominent in many species during fetal development, including the human fetus. However, the intermediate lobe is not present in adult humans suggesting a specific role for the lobe during fetal development.

2.2.4 Ontogeny of POMC gene expression

Whether or not the increase in pituitary ACTH secretion seen in late gestation is accompanied by an increase in POMC gene expression is the subject of much interest. POMC is a polypeptide precursor gene that is processed to produce several biologically active peptides, including ACTH (Eipper and Mains, 1980). The corticotrophs of the anterior lobe and the melanotrophs of the intermediate lobe both express POMC. Differential post-translational processing of the POMC gene results in different peptides being found in these two cell types. ACTH and β-lipotrophin are the major products of corticotrophs while melanotrophs synthesise α-MSH, CLIP and acetylated β-endorphin.

The differential processing of POMC in the anterior and intermediate lobes of the pituitary reflects the expression of POMC processing enzymes, prohormone convertase 1 (PC1) and prohormone convertase 2 (PC2). PC1 and PC2 are expressed exclusively in endocrine and neuroendocrine cells (Seidah, Marcinkiewicz, Benjannet, Gasper, Beaubien, Mattei, Lazure, Mbikay and Chrètien, 1991). In the rodent pituitary PC2 is abundantly expressed in the intermediate lobe whilst PC1 expression dominates in the anterior lobe (Seidah *et al.*, 1991; Hakes,

Birch, Mezey and Dixon, 1991). Cotransfection of POMC with PC1 and/or PC2 into the constitutively secreting cell line BSC-40 and the endocrine tissue-derived cell lines PC12 and AtT-20 has shown that POMC cotransfected with PC1 alone generates the peptides of the anterior pituitary, notably ACTH whilst cotransfection with both PC1 and PC2 generated α-MSH and CLIP characteristic of intermediate lobe melanotrophs (Benjannet, Rondeau, Day, Chretien and Seidah, 1991). The role of PC2 in the additional processing of the POMC molecule in the intermediate pituitary has been demonstrated using the AtT-20 mouse corticotroph cell line. AtT-20 cells transfected with PC2 aquire the ability to perform the additional cleavage of the POMC molecule seen in intermediate pituitary melanotrophs (Zhou, Bloomquit and Mains, 1993).

In the ovine fetus, POMC mRNA has been detected in the pituitary as early as day 60 by Northern analysis (Yang, Challis, Han and Hammond, 1991; McMillen, Mercer and Thornburn, 1988). Reports describing ontogenic changes in POMC mRNA levels measured by Northern analysis in the late gestation ovine fetus have produced conflicting data, with some groups indicating a decline in levels at term (McMillen *et al.*, 1988; Brooks, Currie, Gibson and Thomas, 1992), while others report an increase (Yang *et al.*, 1991; Myers *et al.*, 1993). Myers *et al.* (1993) employed the more sensitive technique of in situ hybridisation to investigate late gestation POMC expression and reported that POMC mRNA levels increase significantly between days 105-107 gestation and days 138-140 gestation. This late gestation increase in POMC gene expression mirrors that of CRH mRNA in the paraventricular nucleus and corresponds with the increase in plasma ACTH concentrations seen at this time. More recently, Matthews *et al.* (1994) have used in situ hybridisation to look at the ontogeny and distribution of POMC gene expression in the developing fetal sheep pituitary. They report that POMC mRNA is detectable by day 60 in both the anterior and the intermediate lobes of the pituitary and describe a regional distribution within the anterior pituitary. Levels of POMC mRNA in the inferior region (the area around the base of the gland) of the anterior pituitary rose during mid and late gestation whilst levels in the superior region (the area around the intermediate lobe) were unchanged throughout gestation. Levels of POMC mRNA in the intermediate lobe were higher than in the anterior and increased significantly in mid gestation.

2.2.5. Maturation of the fetal adrenal gland

The adrenal glands of the fetal sheep are first recognisable at day 28 gestation and by day 60 two distinct zones are apparent in the fetal cortex (Webb, 1980). The fetal adrenal in the sheep has cortical zones similar to that of the adult in that there is no distinct 'fetal zone'. The outer zone has the morphological characteristics of the adult zona glomerulosa by day 80 whilst the inner zone cells remain morphologically immature until day 120 when the innermost cells begin to mature and develop the smooth endoplasmic reticulum and mitochondria with vesicular cristae characteristic of adult zona fasciculata cells (Robinson, Rowe and Wintour, 1979). Progressively more cells of the zona fasciculata mature as gestation proceeds to term. The adrenal glands undergo a rapid phase of hypertrophy and hyperplasia in the last 14 days of gestation, at the same time as fetal plasma levels of cortisol start to rise. During this period the gland doubles in weight. These changes are dependant on ACTH from the fetal pituitary as they can be abolished by fetal hypophysectomy and restored by intrafetal infusion of ACTH (Liggins *et al.*, 1973).

The primate adrenal glands also exhibit a remarkable increase in weight in the final third of gestation (Pepe and Albrecht, 1990). The increase in weight of the primate adrenal late in gestation represents growth of the fetal cortical zone which makes up to 80% of the gland during fetal life. The primate fetal zone lacks 3β-hydroxysteroid dehydrogenase (3β-HSD) activity and therefore secretes mainly dehydroepiandrosterone sulphate (DHAS), the androgenic precursor for placental oestrogens. In the smaller adult zone there is an increase in 3β-HSD activity late in gestation associated with a rise in serum cortisol concentrations.

The capacity of the ovine fetal adrenal to secrete cortisol in response to ACTH follows a triphasic pattern such that the response before day 90 gestation and after day 120 gestation is much greater than in the intervening period of day 90 to 120 gestation (Wintour, Brown, Denton, Hardy, McDougall, Oddie and Whipp, 1975; Glickman and Challis, 1980). The diminished capacity of the fetal adrenal to produce cortisol between day 90 and 120 gestation has been attributed to the very low levels of mRNA encoding the cytochrome P-450 steroid hydroxylase enzymes necessary for cortisol biosynthesis (Tangalatis, Coghlan, Connell, Crawford, Darling, Hammond, Haralambidis, Penschow and Wintour, 1989). These enzymes are ACTH-inducible and the low levels between day 90 and day 120 reflect low

circulating ACTH concentrations at this time. However, if ACTH levels are increased during this period by infusion of exogenous ACTH, the steroidogenic enzymes are activated (Tangalakis, Coghlan, Crawford, Hammond and Wintour, 1990). It is also of interest that this period of reduced cortisol biosynthetic capacity is accompanied by the period of organisation and maturation of the adrenal cortex.

Bilateral adrenalectomy of the fetus during the period of day 90 -day 120 gestation does not influence the circulating concentrations of cortisol in the fetal plasma (Wintour, Coghlan, Hardy, Hennessy, Lingwood and Scoggins, 1980) and it has since been demonstrated that all the cortisol present in the fetal plasma during this period can be accounted for by transplacental passage from the maternal circulation (Hennessy, Coghlan, Hardy, Scoggins and Wintour, 1982). The same workers have shown that as gestation proceeds the contribution of maternal cortisol to the fetal circulation diminishes illustrating the increased activity of the fetal adrenal gland.

2.3. Functional maturation of the HPA axis

The section which follows describes the development HPA function in the fetal sheep. Many of the studies regarding the functionality of the HPA axis have been carried out in adult animals and these will be discussed in the absence of any fetal data. Where data for the fetal sheep is unavailable reference has also been made to work carried out in other species.

2.3.1 Hypothalamic control of ACTH secretion

The critical role of the hypothalamus in the control of ACTH secretion has been demonstrated in hypothalamo-pituitary disconnected fetuses (Antolovich, Clarke, McMillen, Perry, Robinson, Silver and Young, 1990; Antolovich *et al.*, 1991; Ozolins, Young and McMillen, 1992). HPD involves surgical disconnection of the pituitary from the hypothalamus by removal of the neural component of the median eminence above the level of the portal blood supply. In contrast to the previously used technique of pituitary stalk section, HPD does not disturb the blood supply of the pars tuberalis and therefore the amount of infarction following surgery is minimal (Antolovich *et al.*, 1990). Prolactin release in response to thyrotrophin-releasing factor demonstrates the functional integrity of the pituitary following the

surgical procedure and the reduced response to chlorpromazine, a dopamine antagonist, indicates the disruption of hypothalamic input to the pituitary. After HPD, the preparturient surge in fetal ACTH concentrations is completely abolishes but surprisingly there is a significant increase in basal ACTH concentrations (Antolovich *et al.*, 1991) although there is no corresponding increase in plasma cortisol levels. It is possible that this increase in basal ACTH originates from the pars intermedia since this region of the gland undergoes a marked hypertrophy following HPD (Antolovich *et al.*, 1990). Parturition in HPD fetuses is delayed for at least 8 days beyond term demonstrating the need for an intact hypothalamo-pituitary axis in generating the preparturient cortisol rise and the onset of parturition in the fetal sheep. Ozolins *et al.*, 1992 have further addressed the role of an intact hypothalamo-pituitary connection in pituitary-adrenal function and report that HPD prevents the normal pituitary-adrenal response to intrauterine hypoxemia and hypoglycaemia. More recently, Deayton *et al.* (1994) have used HPD at two different time points in late gestation (day 123-127 and day 133-135) to demonstrate that an intact hypothalamo-pituitary connection is required until at least day 135 of gestation for parturition to occur at the correct time point.

Parturition in the fetal sheep can also be delayed by bilateral lesions to the hypothalamic paraventricular nucleus (McDonald and Nathanielsz, 1991). As in HPD fetuses, the preparturient surge in ACTH and cortisol was prevented and the fetuses were not delivered at term suggesting a critical role for the PVN in the sequence of events leading to parturition.

The role of CRH and AVP in the release of ACTH from the anterior pituitary gland and subsequent cortisol release from the fetal adrenal gland has been extensively demonstrated *in vivo* (Brooks, Challis and Norman, 1987; Apostolakis, Longo and Yellon, 1991; Brooks and White, 1990) and *in vitro* (Durand, Cathaird, Dacheux, Naaman and Saez, 1986; Brooks *et al.*, 1987; Lü, Yang and Challis, 1994). The two neuropeptides have been shown to act synergistically (Brooks and White, 1990; Norman and Challis, 1987a; Gillies, Linton and Lowry, 1982) such that concomitant administration of CRH and AVP results in a greater ACTH response than after CRH or AVP administered alone. The relative abilities of CRH and AVP to stimulate ACTH secretion appears to be species specific. In rats, CRH is the main ACTH-releasing factor (Vale, Vaughan, Smith, Yamamoto, Rivier and Rivier, 1983;

Familari, Smith, Smith and Funder, 1989) whereas in sheep AVP is the more potent. Intravenous injection of AVP elicits a greater ACTH response compared with CRH (Pradier, Davicco, Safwate, Tournaire, Dalle, Barlet and Delost, 1986) and *in vitro* responses of sheep anterior pituitary cells to AVP and CRH also reflect the more potent effects of AVP compared to CRH (Familari *et al.*, 1989; Brooks and Gibson, 1992). The response of the fetal pituitary to hypothalamic factors is greater earlier in gestation (Durand *et al.*, 1986), a response that is seen to decline with advancing gestational age (Brooks and White, 1990; Norman and Challis, 1987a). The greater response to AVP in the sheep may reflect the number of receptors present in the anterior pituitary since membrane binding studies have shown that the adult sheep pituitary gland contains twice as many AVP receptors as the rat but only one tenth of the number of CRH receptors (Shen, Clarke, Canny, Funder and Smith, 1990).

The decline in responsiveness of the pituitary to hypothalamic stimulation at a time when ACTH secretion into the fetal plasma is increasing is puzzling. It is possible that the decline in pituitary responsiveness is a function of the increased levels of glucocorticoids in the fetal plasma at this time which negatively feedback to inhibit the pituitary or that the pituitary is desensitised by increasing exposure to hypothalamic stimulation. Desensitisation in response to repeated stimulation with CRH and AVP has been demonstrated in perifusion culture with the ACTH response to repeated pulses of CRH and AVP declining with time (Evans, Brett, McIntosh, McIntosh, McLay, Livesey and Donald, 1988). Desensitisation to each secretogogue occurred independently of the other. Desensitisation induced by repeated stimulation with one secretagogue could be overcome by administration of the other, thus, when the ACTH response to CRH was diminished a single pulse of AVP resulted in a considerably greater response of ACTH. Rat pituitary cells cultures treated with CRH display a reduced response to subsequent stimulation which is accompanied by a reduced ability of CRH to stimulate cAMP production. This implies that desensitisation results from CRH receptor down regulation or uncoupling of the CRH receptor from adenylate cyclase (Hoffman, Ceda and Reisine, 1985). When fetal sheep were given pulses of CRH and/or AVP every 4 hours, there was a decline in ACTH response which was negatively correlated with increased basal concentrations of cortisol. No evidence for desensitisation could be found since dispersed pituitary cells prepared from fetuses treated with CRH pulses in vivo had a greater accumulation of cAMP compared with controls (Brooks *et al.*,

1987). This suggests that stimulated ACTH secretion is tightly regulated by cortisol feedback mechanisms. In support of this hypothesis, Norman and Challis (1987b) found that treatment of day 113-116 fetuses with dexamethasone prior to concomitant injection of CRH plus AVP did not affect the basal values of ACTH but significantly suppressed the CRH plus AVP-stimulated release of ACTH. In day 126-130 fetuses, both basal and stimulated responses were suppressed. This study illustrates the development of glucocorticoid feedback mechanisms influencing pituitary responsiveness in late gestation and suggests that different mechanisms may be employed for the control of basal and stimulated release of ACTH. The number of CRH binding sites in membrane preparations from the fetal sheep anterior pituitary follows a similar time-course to the changes in responsiveness of the pituitary such that the number of binding sites increases from day 70 to reach a maximum at day 125-130 before levels decline towards term (Lü, Yang and Challis, 1991). The reduced number of binding sites may contribute to the reduction in pituitary responsiveness at this time.

Despite the well documented role of CRH and AVP in the release of ACTH from the fetal pituitary, pulsatile infusion of CRH or AVP or a combination of CRH plus AVP does not induce premature delivery (Brooks and White, 1990). In contrast, continuous infusion of a high dose of CRH to fetuses at day 125 gestation has been reported to induce premature delivery (Wintour, Bell, Carson, MacIsaac, Tregear, Vale and Wang, 1986) suggesting that the dose and mode of administration are important factors. However, in this study maternal progesterone concentrations did not decrease in the manner that normally precedes delivery suggesting that the processes of birth in this case are not comparable to the normal situation.

2.3.2. Maturation of adrenal sensitivity to ACTH

As well as increased drive from the hypothalamus to increase circulating ACTH concentrations, the increase in fetal plasma cortisol concentrations late in gestation can also be accounted for by increased adrenal sensitivity to ACTH at this time (Wintour *et al.*, 1975; Manchester and Challis, 1982). Rose *et al.* (1982) examined the effect of injection of $ACTH_{1-24}$ at three time points in gestation on fetal plasma cortisol concentrations and report an increase in adrenal response to ACTH between day 115 gestation and term. The mechanism by which adrenal sensitivity in late gestation is increased has yet to be elucidated however, the correlation with

increasing plasma cortisol levels at this time has led to the suggestion that cortisol itself is involved in the adrenal activation process. Increased cortisol production from fetal adrenal cells, either spontaneously in late gestation (Durand, Cathaird, Morera, Dazard and Saez, 1981) or in response to $ACTH_{1-24}$ (Durand, Cathaird, Locatelli, Dazard and Saez, 1981) is associated with increased production of cAMP. Adrenal cells isolated following concomitant *in vivo* administration of metopirone, an 11β-hydroxylase inhibitor and $ACTH_{1-24}$ do not respond to *in vitro* ACTH challenge with cAMP accumulation and increased cortisol output, as is the case following *in vivo* administration of ACTH alone (Lye and Challis, 1984). Moreover, glucocorticoid administration, together with ACTH plus metopirone restores adrenal cell response to ACTH (Challis, Huhtanen, Sprague, Mitchell and Lye, 1985). The time of increased adrenal responsiveness to ACTH correlates with an increased number of ACTH receptors between day 140 and term (Durand, 1979; Durand, Cathiard and Saez, 1985).

These studies suggest that cortisol functions locally in a paracrine and /or autocrine manner to enhance the action of ACTH on the fetal adrenal. In this way, increasing cortisol levels in late gestation could be positively regulating the sensitivity of the adrenal gland resulting in a further elevation of plasma cortisol levels.

The effect of cortisol on adrenal sensitivity to ACTH is mediated by glucocorticoid receptors (GR) found in the adrenal cortex (Yang and Challis, 1989). The expression of GR in the adrenal cortex is developmentally regulated such that GR number in the adrenal increases significantly at day 100-110, decreases at day 125-130 and increases again at term (Yang and Challis, 1989). Regulation must occur at a post-transcriptional level since the abundance of GR mRNA in the adrenal does not change with gestation (Yang, Hammond and Challis, 1992).

As well as being responsive to ACTH, the fetal adrenal gland may be influenced by other factors. The presence of ACTH-like peptides in the fetal pituitary (Silman *et al.*, 1979) raises the possibility that these peptides may also be trophic to the adrenal gland. Of the POMC-derived peptides of the pituitary, α-MSH from the intermediate lobe has been shown to stimulate cortisol secretion both *in vivo* in fetal sheep, newborn lambs (Llanos, Ramachandran, Creasy, Rudolph and Serón-Ferré, 1979; Glickman, Carson and Challis, 1979) and rabbits (Challis and Torosis, 1977)

and from fetal sheep and human adrenal cells in culture (Glickman *et al.*, 1979; Baird, Kan and Solomon, 1983). This has led to the suggestion that α-MSH is a trophic factor for the adrenal gland and may contribute to the increasing cortisol concentrations seen in the late gestation fetus. Notably, the stimulatory effect of α-MSH on fetal sheep adrenal cells occurs at a time before the gland is responsive to ACTH and could not be reproduced using adult cells (Glickman *et al.*, 1979), suggesting a fetal specific mode of action. α-MSH has been identified in fetal sheep plasma in concentrations which are significantly higher than in the adult (Newman, Wardlaw, Stark, Daniel and Frantz, 1987), again alluding to an intrauterine role. However, whilst this evidence points towards a role for α-MSH in the control of steroidogenesis, there are a number of conflicting reports concerning the action of α-MSH. Glickman *et al.* (1979) report that α-MSH and ACTH are equipotent in stimulating the secretion of cortisol from human fetal adrenal cells. However, Branchault *et al.* (1978) were only able to stimulate cortisol production using α-MSH concentrations 1000 times greater than ACTH. The low potency of α-MSH in eliciting a steroidogenic response from adrenal cells has also been described elsewhere (Baird *et al.*, 1983). In this study, Baird and co-workers report a differential effect of α-MSH on cortisol and dehydroepiandrosterone (DHAS) secretion by human fetal adrenal cells in culture such that high concentrations of α-MSH elicit release of both cortisol and DHAS but lower concentrations stimulate only DHAS secretion suggesting that the intensity of the signal from the pituitary is important in the regulation of adrenal steroidogenesis. α-MSH has also been shown to stimulate DNA synthesis in the fetal adrenal (Rudman, Hollins, Lewis and Chawla, 1980) suggesting a role in adrenal growth.

The fragments resulting from cleavage of the N-terminal portion of the POMC molecule (N-POMC 1-77) have been implicated in adrenal growth in the rat (Estivariz, Iturriza, McLean, Hope and Lowry, 1982). These fragments act as potent stimulators of DNA synthesis and mitogenesis, whilst the intact precursor, N-POMC 1-77 has been shown to potentiate the action of ACTH on adrenal gland RNA synthesis (Al-Dulaili, Williams, Edwards, Salacinski and Lowry, 1982). The possibility that N-terminal fragments of POMC may influence the developing adrenal gland led Saphier *et al.* (1993) to investigate the concentrations of N-terminal POMC and the cleavage product γ3-MSH in the plasma of late gestation fetal sheep. The γ3-MSH fragment has been shown to activate cholesterol ester

hydolase (Pedersen, Brownie and Ling, 1980), an enzyme of the corticosteroid biosynthesis pathway. Saphier and co-workers found relatively high concentrations of N-POMC in the fetal plasma compared to ACTH. Levels of N-POMC declined towards term, coincident with increasing ACTH concentrations, although the concentration of N-POMC on a molar basis was always greater that for ACTH, leading the authors to suggest that much of the N-POMC was of intermediate lobe origin. The decline in N-POMC concentrations was accompanied by an increase in circulating γ3-MSH concentrations suggesting that N-POMC is further processed to its constituent fragment with advancing gestation.

The extent to which POMC-derived peptides other than ACTH are involved in the preparturient cortisol surge remains to be determined. However, the evidence presented above suggests that the fetal intermediate lobe could be of fundamental importance in the maturation of the fetal adrenal and the late gestation drive in adrenal stimulation necessary for the initiation of parturition.

The relative importance of increasing ACTH concentrations as a result of increased hypothalamic drive to the pituitary and increased sensitivity of the adrenal gland to ACTH in the initiation of parturition is unclear. To address this issue, Jacobs *et al.* (1994) administered a constant low dose of ACTH to hypophysectomised fetal lambs to determine whether increasing plasma ACTH concentrations or increased adrenal sensitivity to ACTH was the more potent stimulus for initiation of parturition. The hypophysectomised fetuses receiving low dose ACTH from day 125 gestation delivered at the same time as the intact controls. Hypophysectomised lambs that did not receive the ACTH infusion showed no rise in cortisol and failed to deliver at term. The preparturient rise in cortisol concentrations in the ACTH treated fetuses mimicked that of intact non-infused controls. These data confirm the obligatory role of ACTH in driving cortisol secretion from the fetal adrenal gland but also suggest that an increase in adrenal sensitivity to ACTH in late gestation is important for the preparturient rise in cortisol and the initiation of parturition at the normal time.

Therefore, the fetal adrenal gland displays increased sensitivity to ACTH in late gestation. The mechanism for increased sensitivity is not yet clear however, it is likely that cortisol itself is involved in priming the adrenal to exhibit increased

responsiveness to ACTH (Darbeiba and Durand, 1987; Challis and Hooper, 1989). The contribution of other pituitary factors to adrenal steroidogenesis and their relevance to parturition remains to be determined.

2.4. Glucocorticoid negative feedback

Glucocorticoids from the fetal adrenal gland act through a classical negative feedback loop to influence the HPA axis at the level of both the pituitary and the hypothalamus. Glucocorticoid inhibition of CRH and AVP secretion from the hypothalamus has been demonstrated *in vitro* from hypothalamic neurons (Clarke and Gillies, 1988; Currie, Gillies and Brooks, 1994) and from perifused hypothalami (Brooks *et al.*, 1989). *In vivo*, the negative effects of glucocorticoids have been demonstrated by employing the technique of bilateral adrenalectomy, thereby removing the source of endogenous glucocorticoids and allowing an escape from the constraints of negative feedback. In contrast, exogenous glucocorticoids are often used in whole animal experiments to replace or add to the endogenous hormone. Adrenalectomy of the adult rat results in increased immunostaining for CRH and AVP in the hypothalamus and this increase can be prevented by insertion of glucocorticoid implants around the paraventricular nucleus (Kovacs, Kiss and Makara, 1986). These dexamethasone implanted rats are unable to produce the increased plasma ACTH levels in response to hypotension as seen in control animals. Dexamethasone implants placed near the ovine fetal PVN at day 108-111 gestation also resulted in decreased CRH immunostaining in the PVN and eliminated CRH immunostaining in the median eminence. Similarly, AVP immunostaining in the median eminence was eliminated, although no effect was seen at the level of the PVN (McDonald, Hoffman, Myers and Nathanielsz, 1990).

Glucocorticoid effects on the expression of CRH and AVP mRNA in the PVN has also been investigated. Adrenalectomy of adult rats results in increased expression of prepro-CRH mRNA, the precursor of CRH in the PVN and this effect can be abolished by glucocorticoid replacement (Jingami, Matsukura, Numa and Imura, 1985). Similar effects on AVP mRNA have been reported (Davies, Arentzen, Ried, Manning, Wolfson, Lawrence and Baldino, 1986). Adrenalectomy of ovine fetuses at day 116-121 gestation results in significantly increased levels of CRH gene expression (McMillen, Antolovich, Mercer, Perry and Silver, 1990; Myers, Ding and Nathanielsz, 1991) whilst dexamethasone implants inserted adjacent to the PVN

suppress expression of CRH mRNA (Myers, McDonald, Dunn, Moss and Nathanielsz, 1992). These data illustrate the ability of the fetal PVN to respond to glucocorticoid negative feedback both with altered gene expression and peptide secretion.

The negative feedback regulation of glucocorticoids has also been demonstrated at the level of the pituitary gland. CRH and AVP- induced ACTH secretion in late gestation fetal sheep is suppressed by administration of exogenous glucocorticoids (Norman and Challis, 1987b). Similarly, pre-treatment of pituitary cell cultures from day 125 gestation fetuses with cortisol for 3 days significantly inhibits the ACTH response to CRH stimulation (Brooks, Howe, Porter and Naylor, 1994). Wintour *et al.* (1980) adrenalectomised fetuses at day 90-127 gestation in order to study the feedback mechanism between the adrenal and the pituitary gland and were the first to report an increase in plasma ACTH concentrations following adrenalectomy in the fetal sheep. Elevated plasma ACTH concentrations following adrenalectomy have since been described by others (McMillen *et al.*, 1990; Myers *et al.*, 1991). The glucocorticoid inhibition the pituitary is also reflected in the modulation of POMC mRNA in response to glucocorticoid manipulation. The increase in plasma ACTH concentration following fetal adrenalectomy (Wintour *et al.*, 1980) is accompanied by significantly increased levels of POMC mRNA in the anterior pituitary (McMillen *et al.*, 1990; Myers *et al.*, 1991). Studies in the adult rat have shown that POMC gene expression is increased in response to adrenalectomy and decreased by treatment with dexamethasone (Jingami *et al.*, 1985; Birnberg, Lissitzky, Hinman and Herbert, 1983; Lundblad and Roberts, 1988). Bruhn *et al.* (1984) demonstrated that the increase in POMC mRNA that occurs following adrenalectomy is dependant upon the hypothalamus. The paraventricular nucleus of adrenalectomised rats was destroyed to determine whether the increase in POMC levels following adrenalectomy was dependant upon PVN production of hypothalamic releasing factors. The abundance of POMC mRNA in the PVN ablated rats was significantly lower than adrenalectomised controls illustrating the need for an intact PVN in mediating the increase in POMC mRNA normally associated with adrenalectomy.

Therefore, circulating glucocorticoids can feedback at the level of the hypothalamus and the pituitary. The increases in CRH mRNA following adrenalectomy and the

reduced POMC response in PVN lesioned animals suggests that glucocorticoid effects at the pituitary result from action at the hypothalamus. However, glucocorticoids can suppress anterior pituitary POMC gene expression in hypothalamo-pituitary disconnected adult sheep (Mercer, Clements, Clarke and Funder, 1989) suggesting that glucocorticoid negative feedback can act directly at the level of the pituitary. In support of the direct effects of glucocorticoids on pituitary POMC expression, a specific region of the POMC gene promoter has been identified which contains a binding site for the glucocorticoid receptor (Drouin, Sun and Nemer, 1989). Drouin and co-workers reasoned that the high level of pituitary expression of POMC is likely to involve DNA sequences that confer tissue specificity. They utilised transgenic mice to demonstrate that the 5'-flanking region of the POMC gene was responsible for pituitary specificity (Trembley, Tretjakoff, Peteson, Antakly, Zhang and Drouin, 1988). Glucocorticoids influence gene transcription via specific glucocorticoid response elements (GRE) which bind glucocorticoid receptor. In the case of the POMC gene promoter, the glucocorticoid receptor binding site acts to suppress transcription and has been termed the negative glucocorticoid response element (nGRE : Drouin *et al.*, 1989). It is possible that GR binding to the nGRE prevents the binding of a factor necessary for POMC transcription. To this end, Drouin *et al.* (1989) demonstrated that members of the DNA-binding transcription factor family COUP could effectively compete with GR for the binding site. Thus, it appears that binding of GR to the nGRE of the POMC promoter represses transcription of the POMC gene and therefore provides a mechanism by which glucocorticoids negatively feedback at the level of the pituitary to influence POMC expression. However, the reason why GR should act as an inhibitor to transcription in this case when it is a transcription factor in other tissues remains to be determined.

2.4.1. Overcoming negative feedback

Despite this apparent negative feedback system, ACTH and cortisol concentrations rise concomitantly in the final 15-20 days gestation The mechanism by which the fetus is somehow able to overcome the negative influences of rising glucocorticoid concentrations has been the subject of intense investigation. This section will look at some of the potential explanations for how this phenomenon may occur.

Increased hypothalamic drive

It is likely that a major factor involved in producing increased plasma ACTH concentrations is an upregulation of the hypothalamus such that the drive to the pituitary is increased and is able to overcome the negative feedback effects of rising cortisol concentrations. A definitive answer to this problem can only be provided by direct measurement of CRH and AVP concentrations in the fetal portal blood supply. Despite the success of portal blood sampling in the adult sheep, the technique has yet to be applied to the fetus. As a result of the inability to directly measure hypothalamic secretions to the portal blood, a number of indirect methods have been utilised in an attempt to tackle the problem.

Immunocytochemical staining for the immediate-early gene c-fos as a sign of neuronal activity has demonstrated the stimulation of CRH neurons at the time of parturition (Hoffman, McDonald, Shedwick and Nathanielsz, 1991). At the time of active labour, 70% of the CRH neurons in the PVN expressed c-fos. Interestingly, these workers observed a population of PVN neurons which were activated during labour that did not contain CRH. Whilst there identity remains to be determined, it is likely that they are AVP neurons.

Despite the demonstration of the negative influence of cortisol on CRH and POMC gene expression described above, both CRH and POMC mRNA levels are significantly increased in late gestation (Myers *et al.*, 1993). This increase in CRH gene expression in late gestation is associated with an increase in hypothalamic content of immunoreactive CRH from day 100 gestation to day 135 gestation (Brooks *et al.*, 1989). CRH release from perfused hypothalamic pieces is greater in day 140 gestation pieces compared to day 100 pieces (Brooks *et al.*, 1989) supporting the theory of increased hypothalamic drive. However, dexamethasone inhibition of CRH release was greater at day 140 gestation than at day 100 gestation suggesting, if anything a greater negative feedback effect in late gestation.

In contrast, dexamethasone implants placed directly adjacent to the PVN display a reduced capacity to suppress basal CRH mRNA with increasing gestational age (Myers *et al.*, 1992) thus providing a mechanism whereby continued hypothalamic drive to the pituitary may occur in the presence of elevated cortisol. The mechanisms by which this apparent escape from the constraints of negative feedback

are mediated remains to be determined. CRH neurons have been identified in rats that do not appear to express glucocorticoid receptor (Agnati, Fuxe, Yu, Harfstrand, Okret, Wilkstrom, Goldstein, Soli, Vale and Grustafsson, 1985) suggesting that some CRH neurons are outside the negative feedback loop. Myers *et al.* (1993) identified a subset of CRH mRNA containing neurons that continue to express CRH when the overall expression level has fallen. It would be of interest to discover if this population of neurons are also devoid of glucocorticoid receptors. This subset of CRH neurons could provide the fundamental drive to the pituitary necessary for parturition to occur and certainly, ablation of the paraventricular nucleus in which the CRH neurons reside prevents the preparturient surge in adrenocortical activity (McDonald and Nathanielsz, 1991).

Altered responsiveness to glucocorticoids

Another possible way in which the late gestation fetus overrides the negative feedback influence of cortisol is by altering the sensitivity of the pituitary to circulating cortisol levels. Infusion of increasing concentrations of cortisol to fetal sheep (day 132-142 gestation) does not result in decreased response of nitroprusside-induced ACTH release which one might expect if the negative feedback system was performing as it does earlier in gestation (Wood, 1988). However, fetal sheep of gestational ages between 121 days and 131 days infused with cortisol do show a dose-dependant inhibition of ACTH release (Wood and Rudolph, 1983) suggesting that there is an alteration in the negative feedback threshold at around day 132 gestation. A similar maturation in the response to nitroprusside-induced ACTH release in the presence of cortisol has been demonstrated by others (Hargrave and Rose, 1985; Dix, Rose, Mavis, Hargrave and Meis, 1984). However, the ACTH response to hypotension requires an intact PVN (McDonald, Rose, Figueroa, Gluckman and Nathanielsz, 1988) suggesting that the altered effects of negative feedback described by Wood and others reflects a change in responsiveness at a level above that of the pituitary, such as the PVN. The role of the PVN in the development of cortisol negative feedback has been investigated using HPD fetuses. After day 138 gestation, cortisol infusion significantly decreased basal ACTH concentrations in intact fetuses but had no effect in HPD fetuses (Ozolins, Young and McMillen, 1990). This implies that an intact hypothalamo-pituitary connection is necessary for the development of cortisol negative feedback on basal ACTH concentrations. In contrast, ACTH release in response to CRH was abolished in

both intact and HPD fetuses following cortisol infusion. Therefore, it seems that the negative feedback effects of cortisol on CRH-induced ACTH release can occur directly at the level of the pituitary whilst control of basal ACTH concentrations occurs within the hypothalamus. The study of Ozolins *et al.* (1990) is at odds with that of Wood and co-workers (1988) with respect to the negative influences of cortisol on baseline ACTH values. Whereas Ozolins *et al.* (1990) demonstrate an inhibitory effect of cortisol on basal ACTH concentrations in day 138-140 gestation fetuses, Wood (, 1988) was unable to demonstrate any negative influence of cortisol on basal ACTH concentrations between day 132 and 142 gestation. This is in accordance with the work of Myers *et al.* (1992) who report no effect of dexamethasone implants to the PVN on basal ACTH levels whilst demonstrating a reduction in feedback efficacy with advancing gestational age.

The alteration in responsiveness to glucocorticoids may reflect a change in the expression of glucocorticoid receptors in the developing fetus. The number of glucocorticoid receptors in the fetal pituitary and hypothalamus rises dramatically between day 60-70 and day 100-110 gestation before falling until day 125-130 gestation (Yang, Jones and Challis, 1990). GR number in the pituitary then increases again at term whilst those of the hypothalamus remain unchanged. The abundance of GR mRNA in the pituitary and hypothalamus, measured by Northern blot analysis, does not change throughout gestation (Yang *et al.*, 1992). More recently, in situ hybridisation studies revealed an increase in GR mRNA immediately prior to parturition (Matthews, Yang and Challis, 1995) which was unaffected by cortisol infusion. Thus, glucocorticoids do not directly regulate the expression of GR mRNA in the pituitary and hypothalamus. Perhaps more importantly, the up-regulation of GR mRNA and receptor number at term suggests that GR availability in late gestation is not limited by increasing cortisol levels. These data are therefore inconsistent with the hypothesis of limited GR availability as a mechanism by which the negative feedback of effects of glucocorticoids are reduced.

Modulation of circulating cortisol concentrations

The late gestation increase in cortisol concentrations are mirrored by a parallel increase in the high affinity binding protein for cortisol, corticosteroid binding globulin (CBG) in fetal plasma (Fairclough and Liggins, 1975; Ballard, Kitterman,

Bland, Cylman, Gluckman, Platzker, Kaplan and Grumbach, 1982). A similar increase in CBG can also be in preterm labor induced by pulsatile administration of ACTH (Challis, Nancekievill and Lye, 1985). The major site of CBG production in the fetus is the liver (Berdusco, Yang, Hammond and Challis, 1995) although the fetal pituitary also produces CBG. CBG mRNA in the fetal liver increases significantly from day 125 to day 140 and then declines at term. Glucocorticoid infusion to fetuses increases the abundance of CBG mRNA in the liver and increases the corticosteroid binding capacity of fetal plasma (Berdusco, Hammond, Jacobs, Grolla, Akagi, Langlois and Challis, 1993; Berdusco, Milne and Challis, 1994). Thus, cortisol may regulate its own availability in fetal plasma by modulating the production of CBG and therefore the concentrations of free cortisol in the fetal circulation.

ACTH from another source

The problem of rising plasma ACTH concentrations in the face of elevated cortisol concentrations late in gestation could be accounted for, at least in part if the ACTH in the fetal plasma was to come from a source other than the anterior pituitary. The intermediate lobe of the pituitary is one such possible source. The intermediate lobe of many species is well developed in fetal life and intense immunocytochemical staining for ACTH has been demonstrated in the fetal sheep pars intermedia (Mulvogue *et al.*, 1986; Matthews *et al.*, 1994). Rat neurointermediate lobes in superfusion culture secrete an ACTH-like peptide that is biologically active when added to an adrenal cortical cell suspension (Tilders *et al.*, 1981). Similarly, dispersed pars intermedia cells *in vitro* respond to hypothalamic extracts by releasing an ACTH-like activity (Kraicer and Morris, 1976) which has proved to be high molecular weight ACTH (Kraicer, Elliot and Zimmerman, 1978). More recently, Kraicer *et al.* (1985) have demonstrated the release of bioactive ACTH from perfused rat intermediate lobes in response to CRH. Abundant CRH immunoreactivity has been demonstrated in the neurohypophysis (Burlet, Tonon, Tankosic, Coy and Vaudry, 1983) and this would be an appropriate pathway for hypothalamic AVP and CRH to directly influence the release of intermediate lobe peptides. This pathway is made more likely due to the largely avascular nature of the intermediate lobe. CRH receptors have been identified on the melanotrophs of the intermediate lobe CRH (Aguilera, Millan, Hauger and Catt, 1987; De Souza and Kuhar, 1986). The levels of CRH peptide within the nerve terminals of the neural

lobe are significantly increased by adrenalectomy (Jeandal, VanDorsselaer, Lutz-Butcher and Koch, 1987) and decreased following pituitary stalk section (Saavedra, Rougeot, Culman, Israel, Niwa, Tonon, Vaudry and Dray, 1984). Osmotic stress also results in increased neurointermediate lobe content of CRH (Jessop, Eckland, Todd and Lightman, 1989). Thus it appears that the neurointermediate lobe, of the rat at least contains an ACTH-like factor that is biologically active and is released in response to CRH stimulation. It is interesting to speculate that this intermediate lobe peptide may provide additional drive to the adrenal gland in late gestation thereby contributing to the pre-parturient surge in cortisol.

Thus, there are many ways in which the fetus can overcome the negative feedback influences of cortisol on the HPA axis and evidence exists for possible mechanisms at all levels of the axis. It is likely that no single mechanism exists to overcome negative feedback, but rather the fetus employs a combination of mechanisms, including those described above, in order to escape from the constraints of negative feedback, thus providing the endocrine environment resulting in the onset of parturition.

2.5. Dopaminergic regulation of the pituitary gland

The synthesis and release of pituitary hormones is under a complex hypothalamic regulatory system which comprises both stimulatory and inhibitory factors. The catecholamine, dopamine is a neurotransmitter in the mammalian brain that acts as a neurohormone at the pituitary gland where it conveys a largely inhibitory influence. The section which follows describes the dopaminergic system which influences pituitary function and reviews the role of dopamine in the regulation of prolactin and POMC-derived peptide secretion from the pituitary gland.

2.5.1. Dopaminergic neurons of the hypothalamus

Dopaminergic neurons of the mammalian brain are classified according to the location of their cell bodies and axon terminals. Accordingly, the dopaminergic neurons of the mediobasal hypothalamus are divided into two major groups, the tuberoinfundibular (TIDA) and the tuberohypophysial (THDA) neurons. TIDA neurons originate in the rostro-caudal portion of the arcuate nucleus, corresponding to the A12 cell group of Dahlsröm and Fuxe (1964). The axons of the TIDA

neurons terminate in the external zone of the median eminence where dopamine is released into the portal blood supply. Dopaminergic terminals are abundant in the external zone of the median eminence, where they make up to one third of all terminals (Ben-Jonathan, 1985). THDA neurons also arise in the A12 cell group of the rostral arcuate nucleus, although the distribution of these neurons is less widely spread than that of the TIDA neurons. The THDA neurons project to the neurointermediate lobe of the pituitary. In the neural lobe, the dopamine axon terminals are found in close proximity with magnocellular axon terminals and precapillary spaces and it has been suggested that the dopamine acts to modulate the secretion of the magnocellular hypothalamic hormones from the axons terminating in the posterior pituitary (Holzbauer and Rackè, 1985). The axons innervating the intermediate lobe make close contact with melanocytes. By contrast, the anterior pituitary is highly vascularised but very poorly innervated.

More recent evidence suggests that the dopaminergic neurons terminating in the intermediate lobe do not arise from the arcuate nucleus as was previously thought, but rather they originate in the periventricular nucleus (A14 cell group of Dahlsröm and Fuxe). Injection of the rat neurointermediate lobe with the retrograde tracer horse radish peroxidase results in labelling of tyrosine-hydroxylase immunopositive cells in the periventricular nucleus (Luppi, Sakai, Salvert, Berod and Jouvet, 1986; Kawano and Daikoku, 1987). However, the same technique does not label cells in the arcuate nucleus (Luppi *et al.*, 1986; Kawano and Daikoka, 1987; Kelly and Swanson, 1980). Similarly, tyrosine hydroxylase immunostaining (Davies, Lichtensteiger, Schlumph and Bruink, 1984) and dopamine content (Dawson, Valdes and Annau, 1985) of the pituitary are unaffected following the destruction of dopaminergic neurons of the arcuate nucleus by administration of the neurotoxin monosodium glutamate which causes selective destruction of the retinal and arcuate nuclear neurons (Nemeroff, Konkol, Bissette, Youngblood, Martin, Brazeau, Rone, Prange, Breese and Kizer, 1977). A greater insight into the innervation of intermediate lobe melanocytes comes from the demonstration that surgical isolation of the periventricular nucleus from the mediobasal hypothalamus results in a 50% decrease in the levels of dopamine and the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) extracted from the intermediate lobe (Goudreau, Lindley, Lookingland and Moore, 1992). The decrease in dopamine content is accompanied by an increase in plasma concentrations of α-MSH.

Conversely, plasma α-MSH can be decreased by electrical stimulation of the periventricular nucleus. This study suggests that up to 50% of the dopaminergic neurons innervating the intermediate lobe arise in, or project through the periventricular nucleus. It also demonstrates the negative influence of the dopaminergic neurons on the secretion of α-MSH. Injection of the periventricular nucleus with the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L) and the subsequent analysis of terminals in the intermediate and neural lobes for tyrosine hydroxylase and PHA-L has shown that periventricular dopaminergic neurons innervate the intermediate lobe but not the neural lobe of the pituitary (Goudreau, Falls, Lookingland and Moore, 1995). The possibility that dopaminergic neurons innervating the intermediate lobe arise in the periventricular nucleus rather than the arcuate nucleus as previously thought means that some of the initial observations regarding dopaminergic regulation of the intermediate lobe, particularly those involving ablation of the arcuate nucleus must be interpreted with caution.

Thus, dopaminergic neurons of the mediobasal hypothalamus exist as two distinct populations. The tuberoinfundibular neurons terminate at the level of the median eminence where they release dopamine into the hypophysial portal blood, and are responsible for the dopaminergic regulation of the anterior pituitary gland. By comparison, the tuberohypophysial neurons (or tuberoperiventricular neurons) directly innervate and regulate the neural and intermediate lobes of the pituitary gland.

2.5.2. The dopamine receptor family

The physiological effects of dopamine on the pituitary gland are mediated via dopamine interaction with the pituitary dopamine receptor. The pituitary dopamine receptor is one of a family of multiple dopamine receptor subtypes. Dopamine receptors are classically placed in two distinct categories, D1-like and D2-like receptors (Kebabian and Calne, 1979) according to pharmacological and biochemical criteria. Generally, D1 receptors activate cAMP coupling to second messenger systems and are antagonised by ergot derivatives such as bromocriptine whilst D2 receptors inhibit adenylate cyclase activity and are potently activated by bromocriptine. The knowledge that dopamine receptors were coupled to guanine nucleotide regulatory proteins (G-proteins) (Vallar and Melddesi, 1989) led to their molecular characterisation. Homology screening of the G-protein coupled receptor

superfamily has allowed identification of multiple dopamine receptor subtypes. To date, five structurally different dopamine receptors have been identified using molecular cloning techniques. D3 and D4 receptors are most closely related to D2, although each has a distinct morphological profile and D5 receptor is similar to D1 in that it stimulates adenylate cyclase activity but it has a higher affinity for dopamine. The rat D2 dopamine receptor was identified using homologous sequences to the hamster β-adrenergic receptor, a member of the G-protein coupled receptor superfamily (Bunzow, VanTol, Grandy, Albert, Salon, Christie, Machida, Neve and Civelli, 1988) and was found to be abundant in brain and pituitary tissue. The pituitary D2 receptor can exist as two isoforms (Eidne, Taylor, Zabavnik, Saunders and Inglis, 1989; Giros, Martres, Sokoloff, Riou, Emorine and Schwartz, 1989), a long and a short form that differ by the insertion of 29 amino acids within the third cytoplasmic loop, a region involved in G-protein coupling. Expression of the long receptor form in the adult rat pituitary is 5-10 fold greater than for the short form. The two isoforms display indistinguishable high-affinity for the radiolabelled dopamine antagonist spiroperidol, [^{3}H]spiroperidol and both inhibit adenylate cyclase activity (Dal Toso, Sommer, Ewert, Herb, Pritchett, Bach, Shivers and Seeburg, 1989)

2.5.3. Dopaminergic regulation of POMC-derived peptides

The presence of dopamine receptors on the melanotroph cells of the intermediate lobe has long been inferred from the ability of dopaminergic compounds to influence the release of α-MSH from these cells (Bower, Hadley and Hruby, 1974; Tilders and Mulder, 1975). Electrothermic lesions of the mediobasal hypothalamus of rats encompassing the median eminence demonstrate that the effects of dopamine on the release of α-MSH were occurring directly at the level of the pituitary rather than at the hypothalamus. Treatment with the dopamine agonist bromocriptine prevented the lesion-induced fall in pituitary α-MSH content seen in vehicle treated animals (Tilders and Smelik, 1978), presumably by inhibition of α-MSH secretion from the pituitary. This lead to the conclusion that dopamine delivered to the intermediate lobe can act on pars intermedia melanotrophs directly, rather than influencing α-MSH secretion at the level of the hypothalamus, and suggested that direct innervation of the lobe was likely to be an important mechanism by which dopamine regulates the melanotrophs. The participation of the tuberohypophysial dopaminergic neurons in the inhibitory control of α-MSH secretion was validated

further by the demonstration that dispersed and intact neurointermediate lobes in perifusion culture respond differentially to elevated potassium (Randle, Moor and Kraicer, 1983). Whereas dispersed pars intermedia cells responded with a transient release of α-MSH, intact neurointermediate lobes responded with a sustained decrease in the secretion of α-MSH. This observation led to the suggestion that the effect of potassium on the intact neurointermediate lobes was a result of the dopaminergic neurons within the gland being stimulated to release dopamine, which in turn was acting on the melanotrophs to inhibit the release of peptide. In contrast, potassium acts directly on the dispersed pars intermedia cells to evoke the transient release of α-MSH (Randle *et al.,* 1983). In order to test this hypothesis, the dopamine antagonist d-butaclamol was added to the perifusion system. The finding that d-butaclamol converted the intact neurointermediate lobe response from a sustained decrease to a transient increase in release of α-MSH, characteristic of dispersed cells supports the hypothesis that dopaminergic innervation of the pars intermedia is involved in the inhibitory control of α-MSH from the gland.

Binding of [^{3}H]spiroperidol to rat neurointermediate lobes has identified specific dopamine binding sites (Cronin and Weiner, 1979; Stefanini, Devoto, Marchiso, Vernaleone and Collu, 1980). The binding of [^{3}H]spiroperidol satisfies the criteria for receptor binding, including high affinity binding, saturability and reversibility. Comparison of the properties of spiroperidol binding with the adenylate cyclase diminishing properties of dopamine receptor activity (Frey, Cote, Grewe and Kebabian, 1982) in the intermediate lobe suggest that some or all of the spiroperidol binding sites are D2 receptors, indirectly illustrating the presence of dopamine receptor sites in the intermediate lobe. Treatment of dispersed rat intermediate lobes with dopamine or dopamine agonist inhibits the spontaneous release of α-MSH into the culture medium without altering the basal level of cAMP (Munemura, Eskay and Kebabian, 1980) suggesting that dopaminergic effects on the intermediate lobe are mediated through a D2-like receptor. Further pharmacological characterisation of the dopaminergic effects on melanotrophs (Munemura, Cote, Tsuruta, Eskay and Kebabian, 1980) suggested the receptor fulfilled the criteria of a D2-like dopamine receptor (Kebabian and Calne, 1979). D2 receptor gene expression in the intermediate lobe of the rat pituitary has been demonstrated and expression is dopamine dependant, such that treatment with a dopamine antagonist results in increased expression of D2 receptor mRNA in intermediate lobe cells (Autelitano,

Snyder, Sealfon and Roberts, 1989). Expression of D2 receptor mRNA in the anterior lobe was unaltered by treatment with dopamine antagonist suggesting that the regulation of dopamine receptor mRNA by dopamine is tissue-specific. More recently, an ontogeny study of dopamine receptor during rat fetal development has identified the D2 receptor in the intermediate lobe of the fetal pituitary at embryonic day 17 (René, Hindelang, Stoeckel and Félix, 1994). High levels of expression at day 20 precede dopaminergic innervation of the intermediate lobe by 4-5 days (Gary and Chronwell, 1992).

Dopaminergic regulation of the POMC peptides in the intermediate pituitary occurs as a result of altered gene expression. Intraperitonial injection of the dopamine antagonist haloperidol over a period of 7-21 days significantly increases the amount of [^{3}H]phenylalanine incorporated into isolated neurointermediate lobes and cell-free translation studies demonstrate a 100-150% increase in the amount of translatable mRNA encoding for POMC (Höllt, Haarmaan, Seizinger and Herz, 1982). *In situ* hybridisation studies reveal that individual cells respond to dopaminergic drugs in keeping with the known responses of peptide secretion. Chronic agonist treatment decreases the amount of POMC mRNA, as measured by grain counts to 10% of control values whilst chronic antagonist treatment results in an elevation of grain counts to 300% of control values (Chronwall, Millington, Griffin, Unnerstall and O'Donohue, 1987). In this same study, chronic agonist and antagonist treatment also altered the thickness of the intermediate lobe. The increase in the thickness of the intermediate lobe following antagonist treatment resulted from an increase in the rate of cell proliferation within the lobe as demonstrated by an increase in the level of incorporated [^{3}H]thymidine. The influence of dopaminergic drugs on the size of the intermediate lobe has also been described by others (Beaulieu, Goldman, Miyazaki, Askay, Kebebian and Cote, 1984). The increase in POMC mRNA in the intermediate lobe in response to a specific stress, that of hyperosmosis can be blocked by a D2 receptor antagonist and mimicked by a D2 receptor agonist (Pardy, Carter and Murphy, 1990) illustrating that the decrease in POMC mRNA in response to hyperosmotic stimulation is mediated by dopamine acting through D2 receptors in the intermediate lobe.

The inhibitory effect of bromocriptine on POMC gene expression and peptide secretion can be reversed by pre-treatment with pertussis toxin (Loeffler, Demeneix,

Kley and Höllt, 1988). Pertussis toxin inactivates nucleotide-binding proteins and uncouples the D2 receptor from the regulatory Ni subunit, therefore abolishing the ability of the receptor to inhibit adenylate cyclase activity (Cote, Frey and Sekura, 1984). Pertussis toxin also inactivates another G-protein, No which has been implicated in the control of the Ca^{2+} pathway. Since G-proteins can couple neurotransmitter receptors to specific ion channels and therefore modulate Ca^{2+} entry to the cell (Holz, Rane and Dunlap, 1986), it has been suggested that D2 receptor inhibition of POMC can occur through the Ca^{2+} pathway in addition to the cAMP pathway. In support of this hypothesis, Ca^{2+} has been shown to regulate POMC gene expression in primary cultures of intermediate lobe cells (Loeffler, Kley, Pittius and Höllt, 1986).

Thus, dopaminergic regulation of the intermediate lobe, in the adult at least is well characterised. Dopaminergic innervation of the pars intermedia by tuberohypophysial neurons has been demonstrated and dopaminergic effects on the melanotroph occur through the dopamine D2 receptor which has been localised on pars intermedia melanotrophs. In contrast to the adult, dopaminergic modulation of the fetal intermediate lobe is less widely studied. However, the fetal sheep pituitary is able to respond to dopamine from day 116 gestation (Newman *et al.*, 1987) when an intravenous bolus of the dopamine receptor antagonist metoclopramide results in increased levels of α-MSH in the fetal circulation implying the existence of dopamine receptors in the fetal sheep pituitary gland and illustrating the presence of a functioning dopaminergic system during fetal life.

In contrast to the well documented influence of dopamine on intermediate pituitary secretion, the role of dopamine in the regulation of anterior pituitary ACTH secretion has been less widely investigated. Unlike the melanotrophs of the intermediate lobe, anterior pituitary corticotrophs are not directly innervated by hypothalamic neurons. Thus, any regulatory influence of dopamine on ACTH section would likely occur indirectly through action on CRH and/or AVP secretion from the hypothalamus. In this respect, dopaminergic innervation of the paraventricular nucleus has been demonstrated (Buijs, Geffard, Pool and Hoorneman, 1984; Lindvall, Bjorklund and Skagerberg, 1984) and dopamine has been shown to stimulate the release of both CRH and AVP from adult rat median eminence in vitro (Bridges, Hillhouse and Jones, 1975; Negro-Vilar, 1979). Further

evidence that dopamine acts on hypothalamic factors to influence anterior pituitary ACTH secretion comes from the finding that ACTH secretion in the intact animal is increased by administration of haloperidol (Giraud, Littitsky, Conte-Devolx, Gillioz and Oliver, 1980) but there is no effect of dopaminergic manipulation of anterior pituitary cells in vitro (Giguère, Cotè and Labrie, 1981; Rosa, Policastro and Herbert, 1980). A similar lack of effect on anterior pituitary POMC gene expression has been reported (Levy and Lightman, 1988; Pelltier, 1990; Chen *et al.*, 1983). A 1.5 fold increase in anterior pituitary POMC gene expression in response to haloperidol has been reported by Autelitano and co-workers (Autelitano, Clements, Nikolaidis, Canny and Funder, 1987). The discrepancy in these results may reflect the different periods of exposure. In the study of Autelitano *et al.*, rats were treated for a period of 14 days compared to the maximum of 7 days of treatment used in other studies (Chen *et al.*, 1983; Levy and Lightman, 1988) There was no indication of the duration of treatment in the report by Pelletier (1990).

2.5.4. Dopaminergic regulation of prolactin

In contrast to the sparse data on dopaminergic regulation of POMC-derived peptides, the role of dopamine in the regulation of prolactin secretion from the fetal anterior pituitary has been extensively demonstrated. Prolactin concentrations in fetal plasma increase in late gestation (Winters, Colston, MacDonald and Porter, 1975; Mueller, Gluckman, Kaplan, Rudolph and Grumbach, 1979) and this increase parallels a similar increase in circulating oestrogen levels. Oestrogen has been shown to influence the level of circulating prolactin and it is likely that the increase in plasma prolactin occurs as a result of elevated oestrogen concentrations. Prolactin concentrations in human fetal cord plasma correlate positively with increasing adrenal weight in the human fetus (Winters *et al.*, 1975) during late gestation and fall abruptly post-partum when the adrenal cortex involutes. This lead Winters *et al.* (1975) to suggest that prolactin is trophic to the adrenal gland in fetal life. However, continuous infusion of prolactin to fetal sheep for 10-27 days does not stimulate production of adrenal cortisol (Lowe, Jansen, Thomas and Nathanielsz, 1977) or DHAS (Ballard, Gluckman, Brehier, Kitterman, Rudolph, Kaplan and Grumbach, 1978). Thus, it seems likely that prolactin concentrations in the fetus reflect oestrogen concentrations and that the post-partum decline results from the loss of the rich placental source of these oestrogens.

Dopaminergic regulation of fetal plasma prolactin concentrations has been demonstrated *in vivo*. Intravenous infusion or bolus injection of the dopamine agonists bromocriptine and apomorphine significantly reduces the level of prolactin in the plasma of fetuses at day 118-136 gestation and in neonatal lambs (Gluckman, Marti-Henneberg, Thomsett, Kaplan, Rudolph and Grumbach, 1979). The level of suppression was always greater in the neonates compared to fetuses. This could be a result of the increased concentrations of oestrogen in the fetuses compared to the neonatal lambs since oestradiol has been shown to exhibit potent anti-dopaminergic activity on prolactin secretion from the pituitary (Raymond, Beaulieu, Labrie and Bossier, 1978). Administration of haloperidol, a dopamine antagonist, to fetuses from day 92-140 gestation resulted in elevated plasma prolactin concentrations in fetuses over day 106 gestation, thus demonstrating the presence of an inhibitory dopaminergic pathway. At this stage of gestation fetal plasma prolactin concentrations have not yet begun to increase, suggesting that the high prolactin concentrations are not merely due to an immature dopaminergic regulatory mechanisms. Studies on stalk-sectioned fetuses reveal a dopaminergic influence on prolactin secretion that arises from somewhere other than the hypothalamus, as demonstrated by the persistence of a response to haloperidol after the pituitary has been isolated from the hypothalamus (Gluckman, Leisti, Kaplan and Brumbach, 1983). In this study, the expected rise in plasma prolactin concentrations following release from the inhibitory regulation of dopamine after stalk section of the pituitary does not occur and the authors suggest that an extra-hypothalamic source of dopamine is involved. The source of extra-hypothalamic dopamine influencing pituitary prolactin secretion remains to be determined, although dopamine has been identified in the fetal circulation and amniotic fluid (Ben-Jonathan and Maxson, 1978). More recently, and in contrast to the study of Gluckman *et al.* (1983), hypothalamo-pituitary disconnection of adult sheep resulted in a short term increase in circulating prolactin concentrations. This increase had been attributed to loss of hypothalamic inhibition by dopamine neurons. Removal of posterior pituitary function by electrical lesioning of the hypothalamo-hypophysial tract resulted in a two-fold increase in circulating plasma prolactin concentrations in the days immediately following surgery (Thomas, Cummins, Canny, Rundle, Griffin, Katsahambas and Clarke, 1989) and raises the possibility that the increase in prolactin following HPD occurs as a result of atrophy of the posterior lobe rather than loss of hypothalamic influence. The short portal blood vessels that connect the

posterior and anterior pituitary (Daniels and Pritchard, 1975) provide a functional link whereby the secretions of the posterior pituitary can actively influence anterior pituitary function. The possibility of posterior control over anterior pituitary function will be discussed later (see section 2.6).

Dopamine binding sites in the anterior pituitary have been identified by specific binding of [^{3}H]Spiroperidol and other radiolabelled ligands for the dopamine receptor in a variety of species including rat, sheep steer and cow (Cronin and Weiner, 1979; Stefanini *et al.*, 1980; Cronin, Roberts and Weiner, 1978; Caron, Beaulieu, Raymond, Gagne, Drouin, Lefkowitz and Labrie, 1978; Levant, Grigoriadis and DeSouza, 1993). D2 receptor binding sites in the rat anterior pituitary have been localised specifically to the prolactin-secreting lactotrophs (Goldsmith, Cronin and Weiner, 1979; Schoors, Vauguelin, De Vos, Smets, Velkeniers, Vanhaelst and Dupont, 1991) by combined radioligand binding and immunohistochemistry. The dopaminergic control of prolactin release from lactotrophs has been extensively demonstrated. Dopamine and its agonists specifically inhibit basal adenylate cyclase activity and prolactin release from rat anterior pituitary homogenates, and this inhibition can be blocked by antagonists (Enjalgert and Bockaert, 1982). A similar correlation between dopamine stimulus and inhibition of prolactin secretion is found in intact anterior pituitary cells in culture (Foord, Peters, Dieguez, Scalon and Hall, 1983). Together with effects on secretion of prolactin, dopaminergic stimulation of anterior pituitary cells *in vitro* specifically inhibits prolactin synthesis, as measured by incorporation of [^{35}S]methionine and also results in decreased concentration of prolactin mRNA (Maurer, 1980) suggesting that the effects on prolactin synthesis are ultimately mediated by influencing transcription of prolactin mRNA. Treatment of dispersed pituitary cells with the potent dopaminergic agonist ergocryptine results in significantly decreased hybridisation of a radiolabelled recombinant DNA plasmid (pPRL-2), containing the complete prolactin coding sequence to extracted nuclear RNA samples (Maurer, 1981) suggesting that ergocryptine treatment inhibits prolactin gene transcription. Further, cell-free translation of RNA was significantly decreased by pre-treatment with ergocryptine and this effect could be partially blocked by concomitant cAMP treatment. Treatment with cAMP alone resulted in a rapid stimulation of prolactin gene transcription suggesting that the inhibitory effects of dopamine on prolactin gene transcription are mediated by cAMP.

In addition to the well documented inhibitory role for dopamine in the control of prolactin synthesis and release, evidence also exists to suggest that dopamine can act to stimulate prolactin release. Dopamine administered to rat anterior pituitary monolayer and superfused cultures at concentrations 1000 fold less than those required for inhibition stimulates the release of prolactin by at least 50% when compared to control cultures (Denef, Manet and Dewals, 1980). Pharmacological evidence based on interactions with the D2 receptor agonists and antagonists suggests that the stimulatory effects of dopamine are mediated by a receptor distinct from the D2 receptor (Burris, Stringer and Freeman, 1991). The discovery of D1 and D5 dopamine receptor subtypes that stimulate adenylate cyclase raises the possibility that the stimulatory action of dopamine is mediated by dopamine receptors other than the D2 class of receptors. The role of different dopamine receptor subtypes in the regulation of prolactin secretion can be examined using GH_4C1 cells, which secrete prolactin but do not express dopamine receptors. Transfection of GH_4C1 cells, with either the D2, D1 or D5 receptor and subsequent exposure to dopamine demonstrates that both D1 and D5 receptors are capable of stimulating prolactin release from the transfected cells (Porter, Grandy, Bunzow, Wiles, Civelli and Frawley, 1994). Further, using reverse transcription polymerase chain reaction, the authors were able to identify D5 receptor mRNA in the rat anterior pituitary gland. D1 receptors have also been identified on lactotrophs (Schoors *et al.*, 1991). In this study administration of fenoldopam, a selective D1 receptor agonist resulted in a dose-dependant decrease in prolactin secretion both *in vivo* and *in vitro* without influencing the levels of adenylate cyclase suggesting that D1 receptors are involved in the inhibition rather than stimulation of prolactin release, by providing a non-adenylate cyclase mechanism for inhibiting prolactin secretion. The existence of a dopamine receptor distinct from the D2 receptor that is uncoupled to adenylate cyclase and involved in inhibition of prolactin secretion from rat anterior pituitary lactotrophs has also been inferred from a study utilising antisense oligonucleotides to identify the mRNA species regulating prolactin release (Valerio, Alberici, Tinti, Spano and Memo, 1994). Addition of antisense oligonucleotides that recognise the initiation codon of D2 receptor mRNA to pituitary cultures for 7 days decreased the level of D2 receptor mRNA and the number of [^{3}H]spiroperidol binding sites and also prevented bromocriptine-induced reduction of adenylate cyclase and prolactin mRNA. However, the antisense

oligonucleotide treatment did not affect the bromocriptine-induced inhibition of prolactin release suggesting that the release of prolactin may be regulated by a dopamine receptor that is distinct from the mRNAs encoding the D2 receptor.

The mechanisms by which dopamine receptor stimulation inhibits, or indeed stimulates prolactin secretion remains to be elucidated. It is possible that the D1 receptors described by Schoors *et al.* (1994) are the same as those described by Valerio *et al.* (1994). It is likely that they are linked to an alternative signal transduction pathway and may represent a subset of dopamine receptors distinct from the D1 receptors that classically stimulate adenylate cyclase activity.

2.6. Interactions between the neurointermediate and anterior lobes of the pituitary gland

Recently, much interest has been expressed in the possible role of the neurointermediate lobe in the regulation of prolactin secretion. The existence of short portal blood vessels which link the posterior and anterior pituitary (Daniels and Pritchard, 1975) make it possible for a posterior pituitary derived factor to influence the lactotrophs of the anterior pituitary. Whilst dopamine has been established as the prolactin inhibitory factor, the existence of a prolactin-releasing factor (PRF) has also been alluded to (Shin, 1980; Grosvenor and Mena, 1980). Removal of the posterior lobe of the rat pituitary abolishes the suckling-induced rise in prolactin (Murai and Ben-Jonathan, 1987) and provides evidence for the existence of a PRF within the posterior pituitary which is released in response to suckling. *In vitro* studies utilising perifused anterior pituitary cells have been used to show that posterior pituitary extracts contain potent PRF activity (Hyde, Murai and Ben-Jonathan, 1987). Extracts of mediobasal hypothalamus also contained PRF activity although the amount was significantly less suggesting that the PRF, unlike the other pituitary releasing hormones is preferentially located in the posterior pituitary rather than the hypothalamus. Further characterisation revealed that the PRF could stimulate prolactin secretion in the presence of dopamine and estimation of its molecular weight using 5000 and 1000 molecular weight cut-off membranes, together with treatment with proteolytic enzymes suggest that the PRF is a small peptide (Hyde and Ben-Jonathan, 1988a). This study, together with previous studies

utilising antagonists to oxytocin (Hyde *et al.*, 1987) suggested that the peptide was distinct from all previously characterised prolactin secretagogues.

Posterior pituitary extracts administered via intracarotid injection to adult rats stimulated prolactin release in a dose-dependant fashion (Hyde and Ben-Jonathan, 1988b), illustrating the ability of the PRF to act *in vivo* and in the presence of the tonic dopaminergic inhibitory system. Similar *in vivo* effects of PRF activity in posterior lobe extracts have been described by others (Samson, Martin, Mogg and Fulton, 1990). Dissection of the posterior pituitary into the constituent neural and intermediate lobes revealed that 90% of the PRF activity of the posterior lobe was localised in the intermediate lobe (Laudon, Grossman and Ben-Jonathan, 1990). Murai and Ben-Jonathan (1990) went on to demonstrate that the prolactin surge in ovariectomised rats following oestradiol treatment was dependent on the presence of an intact neurointermediate lobe. The authors concluded that the oestrogen effects on prolactin secretion were mediated by release of a PRF from the neurointermediate lobe which was transported to the anterior pituitary via the short portal blood vessels.

The influence of neurointermediate lobe (NIL) cells on oestrogen-induced prolactin secretion demonstrated *in vivo* by Murai and Ben-Jonathan (1990) has been further studied using *in vitro* co-culture techniques. Co-culture of anterior and posterior pituitary cells for 4 days induces a 2-3 fold increase in the total prolactin cell content, with a significant elevation in prolactin secretion measurable after 8 days of co-culture (Dymshitz and Ben-Jonathan, 1991). The increased release of prolactin into the medium can be mimicked by culture of anterior pituitary cells in posterior pituitary conditioned medium, implicating a releasable factor from the posterior pituitary in the stimulation of prolactin release. The increase in prolactin content and secretion reflects increased prolactin mRNA levels which increase in response to posterior pituitary co-culture (Corcia, Steinmetz, Liy and Ben-Jonathan, 1993). Thymidine incorporation studies suggest that the increased levels of prolactin mRNA, content and secretion do not occur as a result of cell proliferation but rather reflect a stimulation of gene expression within existing lactotrophs. The presence of NIL cells in anterior pituitary cultures exposed to oestradiol increased by 12% the total number of prolactin secreting cells within the culture (Ellerkmann, Nagy and Frawley, 1991). The data of Corcia *et al.* (1993) suggests that this increase is due to

recruitment of existing cells rather than by cell proliferation. The secretory capacity of individual lactotrophs is also augmented by concomitant culture with NIL cells. Moreover, removal of the NIL cells immediately prior to administration of oestradiol completely abolished the oestrogen-induced release of prolactin illustrating the need for continued presence of the NIL cells in mediating the prolactin response. The high abundance of oestrogen receptor mRNA found within the intermediate lobe of the rat pituitary (Pelletier, Liao, Follea and Govindan, 1988) suggests that the PRF released in response to oestrogen is of intermediate origin as opposed to being a neural lobe factor. The finding that dopamine, acting to inhibit α-MSH release prevented the oestradiol-induced recruitment of lactotroph cells and the ability of α-MSH to substitute for both oestradiol and NIL cell presence in the co-culture system (Ellerkman, Nagy and Frawley, 1992) suggests that the prolactin-releasing factor released from the neurointermediate lobe in response to oestradiol stimulation is α-MSH.

Since any factor carried by the short portal blood vessels from the neurointermediate lobe to the anterior lobe preferentially bathes the area closest to the NIL, Porter and Frawley (1992) separated the anterior pituitary into two distinct regions, that which is proximal to the NIL and the remaining area of the gland not in direct contact with the NIL. Dispersed cell cultures of the two distinct areas monitored by reverse haemolytic plaque assay to measure prolactin release. In the control cultures, the outer region of the gland contained the greatest number of prolactin secreting. cells. Treatment of the cells proximal to the NIL with either α-MSH, β-endorphin or oestradiol in the presence of NIL cells resulted in an 8% increase in the number of prolactin secreting cells. No effect was observed in cultures of the outer region of the gland.

The increase in the number of prolactin releasing cells induced by oestradiol treatment in the presence of NIL cells has been shown to involve recruitment of growth hormone secreting sommatotrophs. All newly recruited prolactin secreting cells also secrete growth hormone and the increase in the number of cells releasing both prolactin and growth hormone coincides with a decrease in the number of cells secreting growth hormone alone (Porter, Ellerkmann and Frawley, 1992). This suggests that prolactin recruitment induced by NIL and oestradiol treatment involves induction of prolactin secretion in cells that formerly secreted growth hormone.

Further characterisation of the recruitment of prolactin secreting cells (Porter *et al.*, 1992) reveals that it occurs post-transcriptionally since inhibitors of RNA synthesis do not affect the recruitment of prolactin secreting cells whereas treatment with a protein synthesis inhibitor completely abolishes the recruitment response to NIL and oestradiol treatment. Thus, the oestradiol induced NIL factor appears to induce translation of previously transcribed prolactin mRNA within growth hormone secreting cells to convert them to prolactin secreting cells. The reason for the post-transcriptional block on prolactin synthesis and the mechanisms by which it occurs remain to be elucidated. However, the existence of a store of transcribed prolactin mRNA waiting to be translated provides a rapidly accessible pool of prolactin that is not dependant upon mitosis or transcription of existing prolactin secreting cells. The evidence above suggests a role for α-MSH in the release of prolactin although the mechanisms by which α-MSH overcomes the post-transcriptional block to prolactin synthesis remains to be determined.

Recently, transgenic mice bearing intermediate lobe tumours, induced by ligation of the POMC gene promoter to the simian virus 40 large T antigen have been utilised to study the regulation of prolactin secretion (Allen, Low, Allen and Ben-Jonathan, 1995). Serum prolactin levels were 5 to 6 times higher in mice bearing intermediate lobe tumours compared to control mice. Bioassay of fractionated tumours revealed potent PRF activity which could be separated into two distinct classes, one big PRF molecule with an estimated molecular weight of 70-80 kilodaltons and two small, hydrophobic peptides. Contrary to the evidence reviewed above, the elution profiles of the three PRFs differed from all POMC derived peptides tested, including α-MSH and from other known prolactin secretagogues suggesting that the PRF is indeed produced by melanotrophs of the intermediate lobe but is not α-MSH or any other POMC-derived peptide. Whilst the large PRF producing intermediate lobe tumours of the transgenic mice may provide a useful source for the purification of PRF it is important to note that the transgenic mice do not represent the normal physiological situation, indeed POMC processing in the intermediate lobe tumours is significantly altered (Low, Liu, Hammer, Rubenstein and Allen, 1993).

Scope of this thesis

The overall aim of this thesis was to investigate the neural control of the pituitary gland during fetal development. An initial approach (Chapter 4) utilised primary

cultures of fetal rat hypothalamic neurons in an attempt to monitor the growth and development of fetal AVP neurons and to gain some insight into the factors influencing the functional maturation of these neurons. Later studies, were concerned with the developing fetal pituitary-adrenal axis with particular reference to the pro-opiomelanocortin system during fetal life. The ontogeny of POMC gene expression and expression of the translated peptides ACTH and α-MSH are described in Chapter 5. The main aim of this thesis was to test the hypothesis that POMC-derived peptides from the fetal pars intermedia are subject to tonic dopaminergic regulation and that removal of this tone may result in increased synthesis and release of adrenal active peptides. The approach to this hypothesis was three-fold, to study the effects of dopaminergic manipulation of the fetus on the secretion of POMC-derived peptides and cortisol into the fetal circulation (Chapter 6), on the pituitary content of POMC-derived peptides (Chapter 7) and on the levels of expression of the POMC gene (Chapter 8). Possible differential effects of dopamine on prolactin secretion in summer and winter fetuses was also studied (Chapter 9).

Chapter 3. General materials and methods

This chapter describes the techniques common to a number of studies presented in this thesis. Other methods unique to specific experiments are described in the relevant chapters.

3.1. Sheep.

Sheep of mixed breeds were obtained from the Roslin Institute, Edinburgh and housed at The University of Edinburgh Marshall Building, Roslin, Midlothian. Prior to surgery, sheep were maintained in pens containing between 10-15 animals and fed a constant diet of concentrates twice daily with hay and water given *ad libitum*. Following surgery, sheep were transferred to individual metabolism crates and continued on the same diet throughout the experimental period. Sheep were maintained under natural lighting conditions prior to surgery and artificial light cycles corresponding to natural photoperiod thereafter.

Sheep were time-mated by intravaginal treatment with sponges containing 60mg medroxyprogesterone acetate (Upjohn Ltd, Crawley, Sussex). The sponges were in place for 13 days after which they were withdrawn and 48 hours later ewes were penned with proven rams. The rams were removed after 24 hours and the ewes considered to have an insemination date with day 0 of pregnancy taken as the date upon which the rams were introduced.

3.2. Surgical procedures.

3.2.1 Anaesthesia

Prior to surgery, food but not water was withdrawn for a 24 hour period. Anaesthesia was induced by intravenous injection of pentobarbitone sodium and thiopentane sodium (Sagattal and Intraval respectively; Rhone Mèrieux Ltd., Harlow, Essex) prepared on a per kilogram weight basis. The ewe was placed in the supine position, intubated and maintained with 3-4% halothane (Fluothane, ICI Pharmaceuticals, Macclesfield, Cheshire) in a nitrous oxide/oxygen mixture (~1l/min) using a closed circuit anaesthetic machine before the ewe was secured to the operating table. The abdomen was clipped and cleaned repeatedly with antiseptic solution (Betadine, Napp Laboratories, Cambridge).

3.2.2. Cannulation

A midline incision was made in the skin of the abdomen between the navel and the mammary gland with care taken to avoid the mammary vein. An incision was then made in the peritoneum underlying the midline ridge. A trochar was passed through the flank and sterile catheters inserted through the opening from the exterior. A small incision was made in the uterine wall to expose the hind hooves taking care to avoid the placental cotyledons. The legs of the fetus were then delivered and cannulation of the femoral vein and artery performed. A small incision was made in the skin of the fetus overlying the inner thigh and the femoral vein and artery exposed by blunt dissection with curved forceps. Two sutures were passed under both the artery and vein individually and the most caudal was tied off. A 10cm polyvinyl cannula filled with heparinied saline (100IU/ml) was inserted into the femoral vein and secured in position. An identical cannula was placed in the femoral artery. The cannulae were then secured to underlying muscle and the incision closed. The catheters were further secured by suturing firmly to the skin of the fetus. An additional catheter for sampling amniotic fluid was secured to the rump of the fetus and the fetus repositioned in the body of the uterus. The uterus and underlying amnion were tightly sutured before closing the peritoneum overlying skin layer.

3.2.3. Post-operative care

Ewes made a quick recovery from the surgical procedure and were awake and eating within half an hour of leaving the operating table. Ewes received antibiotics (4ml Depocillan, Glaxovet) two days pre- and two days post-operatively and fetuses were treated with $1x10^6$ penicillan (Crystapen, Glaxovet) administered intravenously and to amniotic fluid for three days post- operatively. Cannulae were attached to a three-way stopcock (Connecta, Viggo AB, Helsinborg, Sweden) and were kept patent between surgery and the start of the experiment by daily flushing with heparinised saline.

Animals were allowed to recover overnight in pens before being moved to metabolism crates for the duration of the experiment.

3.3. Blood sampling and infusions.

Blood samples were withdrawn into a fresh sterile syringe and cannulae were flushed with sterile heparinised saline after each sample. A closed system was used in which the initial volume of blood withdrawn to fill the cannula at each sample point was returned to the fetus to minimise the overall lose of blood volume. Fetal blood gases, pO_2, pCO_2 and pH were monitered in an additional 0.2ml blood withdrawn at various times throughout the course of the experiment using a blood-gas analyser (IL 1306 ; Instrumentation Laboratories, Warrington Cheshire, UK). Blood collected was immediately transfered to heparinised tubes on ice. Samples were spun at 3000rpm in a refrigerated centrifuge for 15 mins and the plasma collected into aliquots which were stored at -20°C until assayed.
Experimental drugs were infused intravenously via a 0.2µm filter (Millipore, Hertfordshire, UK) at a continuous rate of 0.5ml/hour.

3.4. Radioimmunoassays.

All samples were assayed in duplicate and standards and quality controls were assayed in triplicate. Each radioimmunoassay contained at least two standard curves, one at the beginning and one at the end with an additional curve in the middle if required. Each standard curve included three tubes in which standard and antibody were replaced with equal voumes of assay buffer alone to assess non-specific binding (NSB tubes) of the tracer, and three tubes containing antibody but no standard to assess the binding of the antibody to the tracer in the absence of standard (Bo tubes).

3.4.1. α- Melanocyte stimulating hormone

Buffer Phosphate buffered saline (PBS) was prepared by dissolving 8g NaCl in one litre of 0.05M sodium phosphate solution (1 litre 0.5M phosphate buffered stock solution contained 53.25g Na_2HPO_4, 19.5g$NaH_2PO_4.2H_2O$, pH 7.5). Assay buffer consisted of 0.05M PBS containing 0.1% gelatin and 0.01% thiomersal (BDH)

Antibody α-MSH antibody R6FB was the kind gift of Dr. Bridget Baker, University of Bath. The antibody was raised in a rabbit against synthetic α–MSH and has previously been validated for the measurement of α-MSH in sheep plasma (Lincoln and Baker 1995). There was negligable cross-reactivity with ACTH(1-39)

or $CLIP_{(18-39)}$. The antibody was added to the assay at an initial dilution of 1:25,000.

Iodination α–MSH (Cambridge Research Biochemicals, Cambridge, UK) was iodinated using the chloramine T method (Greenwood, Hunter and Glover, 1963). 5μg of α–MSH stored in 10μl 0.5M phosphate buffer was utilised for each iodination. 1mCi $Na^{125}I$ (Amersham International, Aylesbury, Bucks) was added to the hormone and 10μl of chloramine T (1mg/ml in 0.05M phosphate stock) was added to start the reaction. After 15 seconds the reaction was stopped by addition of 25μl sodium metabisulphite (1mg/ml in 0.05M phosphate buffer). The reactant was purified by loading onto a Sep-pak C-18 column (Millipore, Hertfordshire, UK) which had been prewetted sequentially with 2ml 1% trifluoroacetic acid (TFA; Aldrich, Poole, Dorset) in water, 5ml 80% methanol/20% 0.1% TFA and 3ml 0.1% TFA . Free iodine was eluted from the column with 1ml 0.1% TFA in water. The column was then subjected to the following increasing concentrations of methanol (10%, 20%, 30%, 40%, 42%, 43%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 54%, 58%, 60%, and 80%) in 0.1% TFA. Each fraction was counted for 10 seconds in a miniassay gamma counter (Type 6-20, Mini Instruments, Burnham on Crouch, Essex) to determine presence of radiolabelled hormone. Radiolabelled α–MSH eluted from the column as a single peak (Figure 3.1). The fractions containing the highest radioactivity were stored at -20°C in 3ml 25% methanol and 3ml 0.05M phosphate stock containing 0.25% bovine serum albumin (BSA, Sigma Chemicals, Poole, Dorset : RIA grade, fraction V) and 0.1% mercaptoethanol (Sigma). The fraction which showed the highest binding to the α–MSH antiserum was used for asssay. Radiolabelled tracer was used at 15000cpm in 50μl assay buffer per tube.

Standards and Quality controls α–MSH used for standards was the same as that used for iodination. Standards in the range 8000pg/ml to 30 pg/ml were prepared by double diluting in α–MSH assay buffer from an 8000pg/ml stock. Results are expressed as pg α–MSH/ml. Quality controls consisted of α-MSH added to dexamethasone suppressed sheep plasma at three different dilutions.

Separation Bound hormone was separated by second antibody precipitation using normal rabbit serum (1:400) and donkey anti rabbit serum (1:16, both from The Scottish Antibody Production Unit, SAPU).

Method 100μl of standard, sample or quality control and 200μl assay buffer was added to a 3ml polystyrene tube. Standards and QCs were assayed in

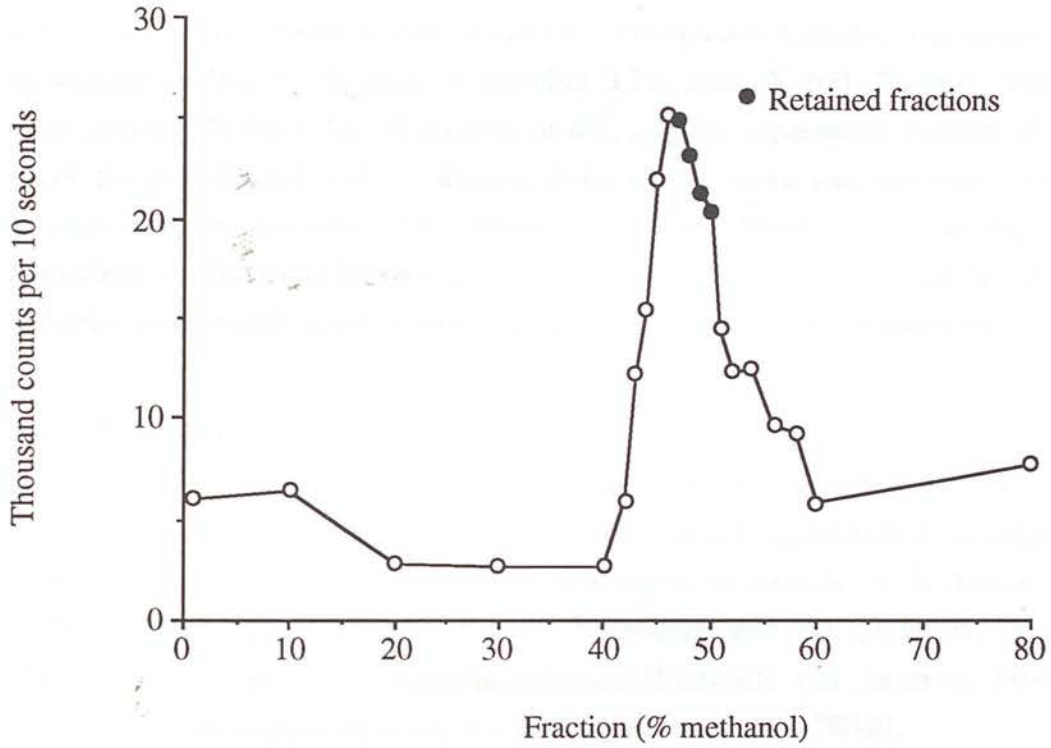


Figure 3.1. Radioactivity in fractions eluted from Sep-pak C-18 column after radioiodination of α-MSH. Dark circles show those fractions that were retained for binding analysis.

triplicate and samples were assayed in duplicate. 50µl of diluted antiserum (1:25000 in assay buffer) was added to the tubes which were vortexed and incubated overnight at 4°C. After an 18 hour incubation at 4°C, 50µl of radiolabelled tracer (15000cpm/50µl) was added to each tube and incubated overnight at 4°C. On the third day 100µl normal rabbit serum and 100µl donkey anti-rabbit serum were added to the tubes to precipitate bound hormone and the tubes were again incubated overnight at 4°C. Precipitated hormone was separated by addition of 1ml 0.9% saline containing 0.2% triton X-100 (Sigma). Tubes were spun at 3000rpm for 30 minutes at 4°C and the supernatent poured off to leave the precipitated pellet. Radioactivity in the tubes was counted for 60 seconds in a gamma counter (1261 Multigamma , LKB, Wallac OY, Finland)

Sensitivity - The assay had a lower limit of detection of 32 pg/ml and the intra- and inter-assay coefficients of variation were 12.7% and 12.5% respectively.

3.4.2. Prolactin

Buffer Assay buffer consisted of 1% BSA in 0.075M phosphate buffered saline(150ml 0.5M phosphate buffer stock, 8.77g NaCl, 0.1g thiomersal per litre)

Antibody Prolactin antibody (#50) was raised in rabbits by Professor A. McNeilly, Reproductive Biology Unit, Edinburgh and has previously been characterised for prolactin radioimmunoassay (McNeilly and Andrews, 1974). The antiserum is used in the assay at a working dilution of 1:128000.

Iodination Purified ovine prolactin, NIADDK-oPRL-19 (NIH) was iodinated using the lactoperoxidase method. 5µl (500µCi) of $Na^{125}I$ (Amersham International) and 5µg lactoperoxidase (Sigma) in 10µl thiomersal-free BSA were added to 5µg purified ovine prolactin (NIADDK-oPRL-19, NIH). 10ml hydrogen peroxide (10µlH_2O_2/150 ml distilled water was added to start the reaction. After 20 seconds, the reaction was stopped by addition of 10µl of a 1mg/ml solution of 2-mercaptoethylammonium chloride in distilled water. 1ml of thiomersal-free 1%BSA/PBS was added to increase the volume of the reaction mixture. The resulting mixture was subjected to gel filtration on a Sephadex G-100 (Pharmacia AB, Uppsala, Sweden) column. The gel was allowed to swell in thiomersal-free BSA that had first been allowed to equilibrate to room temperature. After the column was poured and allowed to pack down, it was coated with thiomersal-free 1%BSA/PBS. The iodination mixture was added to the column and eluted with 1%BSA/PBS. The eluate from the column was collected in 8ml polysterene tubes

using a programmable electronic fraction collector (Gilson model 203, Middleton, WI, USA) and the radioactivity in each fraction monitored (Mini-assay type 6-20, Mini Instruments, Burnham-on-Crouch, Essex). The first peak eluted from the column was radiolabelled oPRL tracer (Figure 3.2). The fractions from this peak containing the highest radioactivity were pooled and stored in 200µl aliquots at -20°C until required.

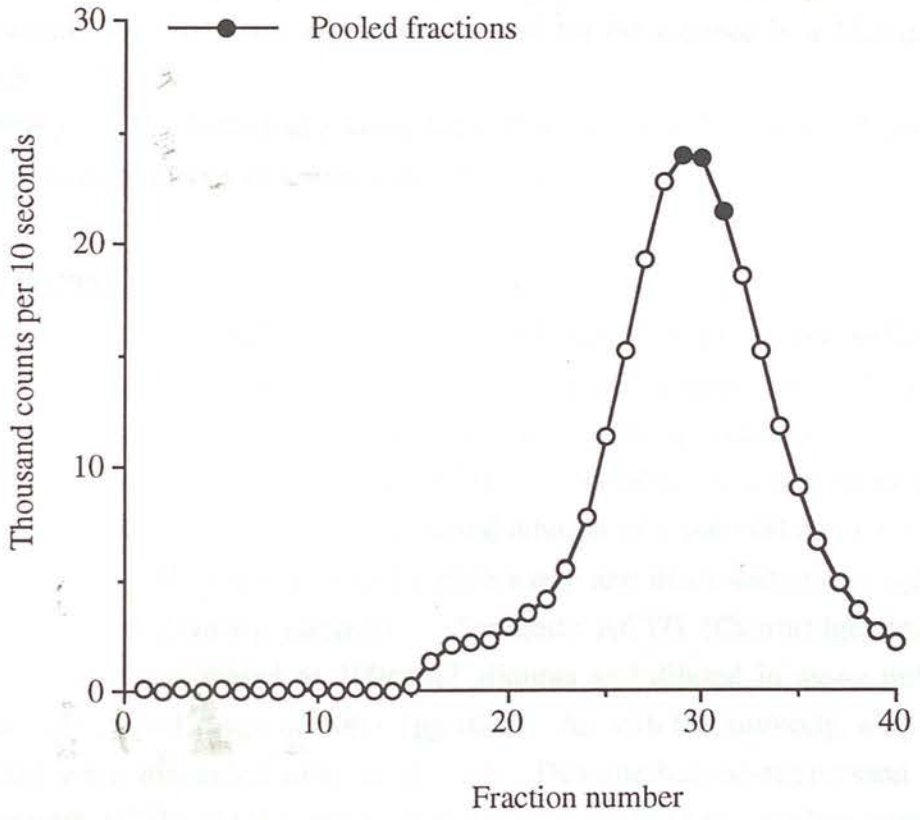


Figure 3.2. Radioactivity in fractions eluted from column after radioiodination of prolactin. Dark circles show those fractions that were pooled and subsequently used in radioimmunoassay.

Standards and Quality controls Prolactin standard (NIH-S15) was stored as 100µg/ml aliquots and diluted in assay buffer to produce standards in the range 200-0.8 ng/ml. Dexamethasone-suppressed sheep plasma with oPRL standard added at three different concentrations was used as a quality control.

Separation Separation was by the second antibody precipitation method using normal rabbit serum (1:400) and donkey anti-rabbit serum (1:16: both from SAPU).

Method 30µl of sample (diluted if required) or standard was added to 100µl assay buffer, 100µl prolactin antibody and 100µl radiolabelled prolactin and incubated overnight at 4°C. The next day 100µl NRS and 100µl DARS, was added to the tubes which were vortexed and returned to 4°C overnight. 1ml of 0.9% saline was added and the tubes spun at 3000rpm for 30 minutes at 4°C. The supernatant was discarded and tubes counted for 60 seconds in a Multigamma counter.

Sensitivity - The assay had a lower limit of detection of 0.6ng/ml and intra- and inter-assay coefficients of variation of 8.5% and 10.2% respecively.

3.4.3. ACTH

Buffer Assay buffer consisted of 0.4% EDTA in phosphate buffer with 0.02% sodium azide; pH 7.4. On the day of use 1µl/ml Triton X-100 and 250KIU/ml aprotinin (Sigma) was added to the stock buffer solution.

Antibody ACTH antibody (AFP6328031; NIDDK) was stored in frozen aliquots and added to the assay at an initial dilution of 1:100,000 diluted in assay buffer. A fresh aliquot was used for each assay and discarded after single use.

Standards and Quality controls Synthetic ACTH (Cambridge Research Biochemicals) was stored as 100ng/ml aliquots and diluted in assay buffer to produce a standard range of 500 - 1pg/100ul. As with the antibody, aliquots of standard were discarded after single use. Dexamethasone-suppressed sheep plasma with ACTH added at three concentrations was used as a quality control.

Iodination 2µg ACTH in 5µl 0.01M HCl was iodinated by the chloramineT method as described above. The resultant mixture was purified by gel filtration on a Sephadex G50 column (Pharmacia AB, Uppsala, Sweden). The Sephadex gel was allowed to swell in assay buffer and was packed into a 50 x 1cm glass column (Amicon Ltd., Stonehouse, Gloustershire). The column was then coated with 10ml 5% BSA. The iodination reaction was loaded onto the column and eluted in assay buffer. The eluate was collected as 1ml fractions in 8ml polystyrene tubes using a programmable electronic fraction collector (Gilson model 203, Middleton, WI, USA) and the radioactivity in each fraction monitored. Radiolabeled ACTH

eluted from the column as a single peak (Figure 3.3) and appropriate fractions were pooled and stored in aliquots at -20°C until used for radioimmunoassay.

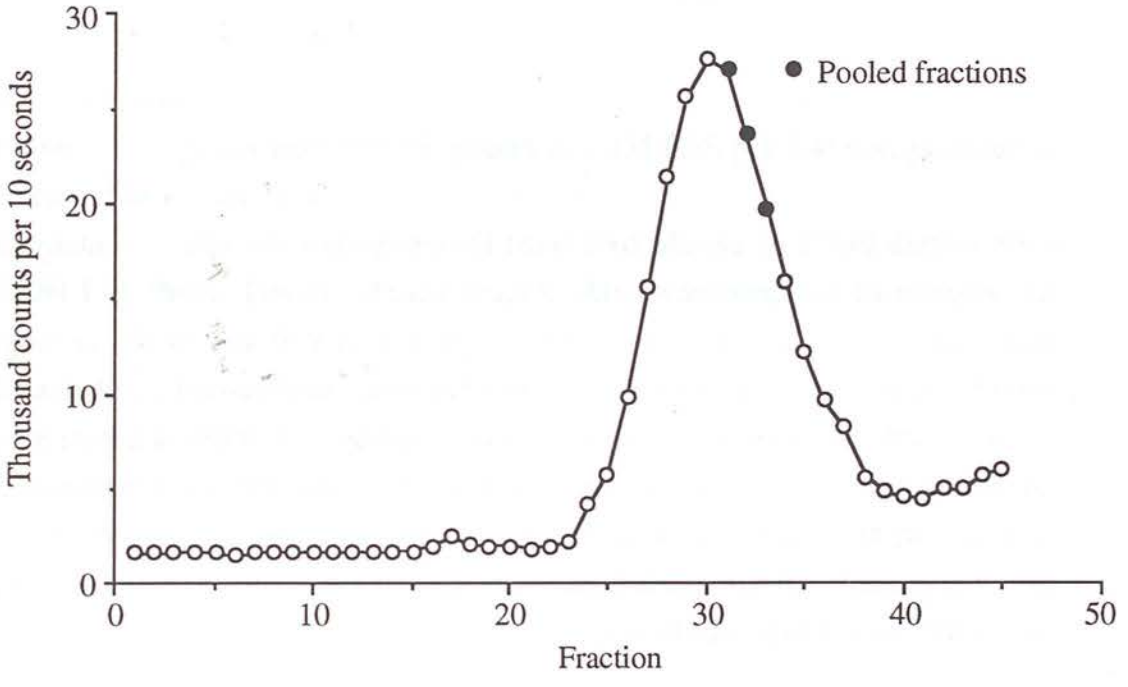


Figure 3.4. Radioactivity in fractions eluted from Sephadex G-100 column after radioiodination of ACTH. Dark circles represent fractions that were pooled and used as ^{125}I-ACTH tracer.

Separation Bound hormone was separated by 30 minute incubation with 500µl donkey anti-rabbit IgG magnetic particles (gift of Dr Rodney Kelly, MRC Reproductive Biology Unit) diluted 1:80 in 0.05M phosphate buffer containing 0.015% Triton X-100. Tubes were vortexed and placed onto magnetic separation racks (Amersham International). After 15 minutes, the supernatant was decanted and the magnet was removed. 300µl of wash buffer (0.075M PBS, 0.01% thiomersal) was added to the tubes before returning to the magnet for a further 15 minutes. Supernatant was decanted and the tubes left inverted to drain for 30 minutes. The radioactivity in the pellet was counted for 60 seconds.

Method 100µl of ACTH antiserum diluted 1:100K was added to 100µl standard, sample or quality control in a 3ml polypropelene tube and incubated

overnight at 4°C. The next day 100µl of radioladelled ^{125}I ACTH was added to each tube (15000cpm/100µl), tubes were vortexed and incubated overnight at 4°C. On the third day, bound antibody was separated by magnetic separation as described above and tubes counted for 60 seconds in a gamma counter.

3.4.4. Cortisol

Buffer Assay buffer (0.1% gelatin in 0.1M PBS pH 7.4) was prepared as described in section 3.4.1.

Extraction Steroids were extracted from 50µl plasma in 2.5ml diethyl ether (BDH Ltd, Poole, Dorset : Analar grade). After vortexing for 10 minutes, the aqueous phase was frozen in a dry ice/methanol bath and the organic phase decanted into boroscilicate tubes and evaporated under nitrogen. The dried tubes were stored at -20°C and reconstituted in 250µl assay buffer at the time of assay. Reconstituted samples were vortexed and allowed to stand at room temperature for 30 minutes prior to assay. Each batch of samples were extracted in parallel with six 100µl aliquots of dexamethasone suppressed sheep plasma containing a 10µl aliqout of ^{125}I cortisol to calculate the efficiency of each extraction. The mean extraction efficiency was 95%

Antibody Anti-cortisol S004-201 (SAPU) was raised in sheep against cortisol-3-(carboxymethyl)-oxime-BSA conjugate. Aliquots of antibody were diluted 1:8000 in assay buffer prior to use. Antibody crossreativity as quoted by SAPU are: corticosterone, 0.18%; cortisone, 0.07%; 21-deoxycortisone, 0.30%; 11-deoxycorticosterone, 0.03%; 17α-progesterone, 2.10%.

Iodination ^{125}I-Cortisol (cortisol-3-(carboxymethyl)-oximino-(2-[^{125}I]-iodohistamine) was purchased from Amersham International and used at 15000cpm/100µl.

Standards and Quality controls Cortisol standard (Sigma) was stored as a 1mg/ml solution in ethanol in a glass vial at -20°C. From the top stock solution a working stock of 100µg/10ml was prepared in assay buffer and stored at 4°C. Standards for assay in the range 8000-15.6pg/100ul were prepared on the day of use from the 4°C stock. Dexamethasone-suppressed sheep plasma with cortisol added at three concentrations was used as quality controls.

Separation Separation was by the second antibody precipitation method using normal sheep serum (NSS, 1:600) and donkey anti-sheep serum (DASS, 1:30, both from SAPU) diluted in assay buffer.

Method On day one, 100µl aliqouts of extracted sample were incubated with 100µl anti-cortisol antibody and 100µl of the radiolabelled tracer for at least 4 hours at room temperature. After the incubation period, all tubes except the total counts received 100µl NSS and 100µl DASS and the tubes were incubated overnight at 4°C. The next day, 1ml 0.9% saline containing 0.2% Triton X-100 was added to all tubes except total counts and the tubes spun at 2500rpm for 30 minutes at 4°C. The supernatant was decanted and the tubes counted for 60 seconds in a Multigamma counter. Results were corrected for extraction efficiency by reference to the control extraction samples.

Sensitivity The assay had a lower limit of detection of 0.5ng/ml and intra- and inter-assay coefficients of variation of 13% and 15% respectively.

3.5. ACTH Immunoradiometric assay.

Buffer Assay buffer was made by addition of 5g BSA, 1ml TritonX-100 and 0.01g sodium azide to 1 litre of 0.1M phosphate buffer.

Tracer/antibody Iodinated ACTH antibodies were purchased from Europath Ltd, Cornwall, UK. Lyophilised vials were reconstituted on the day of use according to the manufacturers instructions.

Standards Synthetic $ACTH_{1-39}$ (Cambridge Research Biochemicals) standard was stored as 100ng/ml aliquots in 0.05M phosphate buffer, 0.5% BSA, 0.02% sodium azide. A fresh aliquot was used for each assay and was diluted in assay buffer to give a standard range of 5000-5 pg/ml.

Separation Bound hormone was separated by second antibody precipitation using normal rabbit serum (NRS, 1:400) and donkey anti-rabbit serum (DARS, 1:20, SAPU).

Method Standard curves were set up as described previously except for the addition of 100µl dexamethasone suppressed plasma to all standard and Bo tubes. NSB tubes were not included since the radiolabelled tracer was also the antibody. 100µl of sample, 100µl assay buffer and 100µl of the labelled antibody cocktail were incubated overnight at 4°C. The next day 100µl NRS (1:400) and 100µl DARS (1:20) was added to all tubes except total counts and the tubes were left to incubate for 4 hours at room temperature. 1ml of 2.5% polyethylene glycol 600 (BDH Ltd) in 0.9% saline was added to each tube before spinning at 3000rpm for 30 minutes at 4°C. Tubes were inverted and drained for 30 minutes before counting for 60 seconds in a Multigamma counter.

Sensitivity The assay had a lower limit of detection of 15pg/ml and the intera- and inter-assay coefficients of variation were 9.8% and 6.2% respectively.

3.6. Tissue collection and processing.

3.6.1. Collection to paraformaldehyde

Ewes were killed with an intravenous overdose of anaesthetic (Euthasate; Willows Fransic Veterinary, Crawley, Sussex) and fetuses were removed through an abdominal incision. Fetuses received an anaesthetic overdose in the form of a cardiac puncture and intact fetal pituitaries were collected immediately. Tissues were fixed for 24 hours in 4% paraformaldehyde (BDH Ltd.) in phosphate buffered saline before transferring to 70% ethanol for temporary storage before processing.

3.6.2. Processing and sectioning of fixed tissue

Tissue was processed through a graded series of alcohols in an automatic 2LE Processor (Shandon Scientific Limited, Cheshire, England) using a standard 17.5 hour cycle and embedded in paraffin wax.

Glass microscope slides to be used for *in situ* hybridisation were washed, dried and baked at 300°C for 8h. Slides were then rinsed sequentially in acetone (BDH Ltd.), 0.25% (v/v) 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone (BDH Ltd.) and filtered distilled water before air drying.

Paraffin wax embedded tissue was sectioned to a thickness of 5µm using a hand operated Microtome (Jung RM 2035, Leica). Sections for *in situ* hybridisation were floated on RNase-free water (double-distilled water UV crosslinked to inhibit RNase activity), transferred onto the treated slides and dried overnight at 50°C before use. Sections for use in immunostaining were sectioned as above but were floated on distilled water before drying onto treated slides.

3.6.3. Collection to liquid nitrogen

The intermediate lobe of the pituitary gland was dissected free of the anterior lobe, weighed and frozen in liquid nitrogen. A small coronal slice, 2-3mm, was removed from the middle of the anterior lobe and placed in 4% paraformaldehyde and was processed for sectioning as described above. The remaining two pieces of anterior lobe were cut in half along the midline/intermediate lobe tract and opposing quarters were weighed and frozen together in liquid nitrogen.

3.7. Immunohistochemistry.

3.7.1. Antibodies

Three different antibodies for immunohistochemistry were routinely used in these studies.

(i) anti- α- melanocyte stimulating hormone (R6FB).

R6FB was the kind gift of Dr. Bridget Baker University of Bath and is the same antibody used in the radioimmunoassay studies in Chapter 6. α–MSH antibody was used for immunohistochemistry at a concentration of 1:1000.

(ii) prolactin antibody #50.

The prolactin antibody #50 was raised in a rabbit and was the kind gift of Prof. A McNeilly, MRC Reproductive Biology Unit, Edinburgh. Prolactin #50 was the same antibody used in the prolactin radioimunoassay studies in Chapter 6. The antibody was used at a concentration of 1:20000 for immunohistochemisty.

(iii) corticotrophin-like intermediate lobe peptide (CLIP) antibody.

The CLIP antibody was a gift from Professor L.Rees, Dept. of Clinical Endocrinology, St.Bartholomews Hospital, London. This is a rabbit antibody raised against $ACTH_{(18-39)}$ which also shows 100% cross-reactivity with $ACTH_{(1-39)}$. CLIP antibody was used at a dilution of 1:500.

The optimal concentration for all primary antibodies was determined by testing various dilutions of each antibody in the immunohistochemical procedure outlined below. The optimal dilution was identified as the dilution that resulted in specific staining with the minimum amount of background staining. Specificity of the antibody staining was assessed by preabsorption of the antibody for 24 hours at 4°C with 5μM concentrations of the antigen. The preabsorbed antibody was then treated as a primary antibody in the method outlined below.

3.7.2. Tissue pretreatment and primary antibody

Tissue prepared as described in section 3.6 was dewaxed in histoclear (National Diagnostics, Atlanta, Georgia, USA) for 15 mins and rehydrated through an alcohol series to water. Endogenous peroxidase activity within the tissue was inhibited by a 30 mins incubation in a solution of 3% (v/v) hydrogen peroxide (Sigma) in methanol (BDH Ltd.). Slides were then washed in 0.5% triton X-100 in water for 15 mins to increase antibody penetration followed by two 5 minute

washes in Tris buffered saline pH7.6 (TBS ; 0.9% NaCl in 0.05M Tris buffer). Tris buffered saline was used in washes to reduce non-specific ionic interactions of the tissue with antibody. Slides were then treated with a blocking solution containing 5% (w/v) bovine serum albumin diluted in 25% normal swine serum in TBS and incubated in a humidity chamber for 30 mins at room temperature. The blocking solution was removed and replaced with primary antibody diluted to the required concentration in blocking solution and incubated overnight in the humidity chamber at 4°C.

3.7.3. Secondary antibody and detection system

Horse radish peroxidase (HRP) detection system. The secondary antibody for the HRP detection system was biotinylated swine anti-rabbit immunoglobulins (SARB; Sigma). After removal of primary antibody with two washes in TBS, SARB diluted 1:500 in TBS was added to the tissue sections and incubated in a humidity chamber for 30min. Excess antibody was removed by 2 x 5min washes with TBS. Sections were incubated with an AB-HRP complex (Dako, Glostrup, Denmark) for 30min. This is a solution of avidin and biotinylated-HRP which serves to amplify the signal obtained from the primary antibody. The solution was prepared according to the suppliers instructions in 0.05M Tris/HCl pH7.6 at least 20min before use. Excess AB-HRP complex was removed by 2 x 5min washes in TBS and bound antibody visualised with a solution of 225µM diaminobenzidine (Sigma) in 0.05M Tris/HCl, pH7.6 containing 0.01% hydrogen peroxide. The slides were monitered microscopically until the colour reaction developed and the reaction terminated by rinsing in water. Slides were then counterstained in haematoxylin, dehydrated through the alcohol series and mounted with Pretex (Cell Path PLC, Hemel Hempstead, Herts., England).

3.8. RNA extraction and separation.

3.8.1. RNA extraction

RNA was extracted from frozen tissue according to the method of Chomczynski & Sacchi (1987) with appropriate adjustment of volumes depending on tissue weight. The tissue was homogenised in solution D which consisted of 4M guanidine thiocynate (Fluka Biochemika, Gillingham, Dorset, UK), 25mM sodium citrate

(Sigma), 0.5% sarcosyl (Sigma) and 0.1M β-mercaptoethanol (Sigma). Tissue was homogenised for approximately 30secs until completely dissociated. To this homogenate was added 2ml 2M sodium acetate (Sigma) pH4.0, 20ml water saturated phenol (Gibco BRL, Paisley, Scotland) and 4ml chloroform/isoamyl alcohol (v/v 49:1, Sigma). The solution was mixed vigorously by shaking after each addition. The mixture was placed on ice for 15min and then centrifuged at 10,000g for 20min at 4°C. The top, aqueous phase containing the RNA was removed to a new tube and an equal volume of cold (-20°C) isopropanol (Sigma) was added. The RNA was precipitated at -20°C for at least 1h, centrifuged at 10,000g for 20min at 4°C, the supernatant was discarded and the RNA pellet redissolved in approximately 1ml solution D. An equal volume of isopropanol was added and the RNA reprecipitated at -20°C for at least 1h. RNA was pelleted by centrifugation (10,000g for 20min), washed in 1ml 75% ethanol, recentrifuged and air dried. The RNA pellet was dissolved in RNase-free water by warming to 65°C for 10min and stored at -70°C.

RNA was scanned at 260 and 280nm on a spectrophotometer. The 260:280 ratio for each sample was calculated to give an estimation of the purity of the RNA. A ratio of 2.0 was taken to be pure. The concentration of the RNA was calculated from the 260nm value where an optical density of 1.0 is equal to 40µg/ml RNA.

3.8.2. Separation of RNA on denaturing agarose gels

RNA was separated on a 1.5% denaturing agarose gel. The gel was prepared by melting 2.25g Seakem agarose (Flowgen, Sittingbourne, England) in 127ml RNase-free water. This was cooled to about 60°C and 15ml of 10 x running buffer (consisting of 200mM 3-[N-Morpholino]propanesulfonic acid (MOPS), 10mM EDTA and 50mM sodium acetate, pH7.0) plus 8.1ml 37% formaldehyde (all from Sigma) added. The solution was mixed gently and poured into a gel tray (15 x 20cm) containing a 15 well comb in a fume hood. After setting, the comb was removed and the gel submerged in 1 x running buffer in a Sub-Cell electrophoresis cell (Bio-Rad, Hertfordshire, England).

RNA (15µg) in a volume of no greater than 5µl was prepared by adding 15.6µl of sample buffer and heating at 60°C for 5min. Sample buffer consisted of 100µl 10 x running buffer, 500µl deionised formamide and 178µl formaldehyde. After

heating, 8µl of dye solution containing 7.5% (w/v) ficoll 400, 0.1% w/v bromophenol blue and 1µl of 1mg/ml ethidium bromide (all from Sigma) was added to each sample. RNA was loaded into individual wells of the gel and separated by running overnight at 35V or for 5hours at 120V.

3.8.3. Concentration of RNA samples by ethanol precipitation

For RNA samples that required concentration, one tenth of the sample volume of 3M sodium acetate (pH 5.5 ; Sigma) was added . Two volumes of cold (-20°C) absolute alcohol (Sigma) was added and the sample left to precipitate overnight at -20°C. The next day, the sample was centrifuged for 20 minutes at 13000rpm, supernatant decanted and the RNA pellet redissolved in RNase-free water.

3.8.4. RNA transfer to membrane

RNA separated on a denaturing gel (section 3.8.2) was transferred to nylon membrane (Hybond-N; Amersham International) by capillary blotting as shown in Fig 3.4. The nylon membrane was prewetted with RNase-free water followed by 20 x SSC (175.3g naCl, 88.2g sodium citrate in 1 litre RNase-free water: pH 7) before placing carefully on the gel. Transfer using 20 x SSC was allowed to continue for a minimum of 12h. After transfer the position of the gel wells were marked on the membrane and RNA was bound to the membrane by exposure to UV light. Membranes were wrapped in cling film and stored at 4°C until used for Northern analysis (Section 3.10).

3.9. Plasmid preparation and analysis.

3.9.1. Transformation of POMC plasmids into competent cells

One 40µl vial of competent cells (XL-1 blue; Stratagene Ltd., Cambridge, UK), 800 µl SOC medium (0.5% Bacto yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) and POMC sense and antisense plasmid in TE buffer (consisting of 10mM Tris-HCl, pH8, 1mM EDTA) were thawed on ice. 1µl (5-10ng) of POMC probe (sense or antisense) was added to an aliquot of competent cells. The tube was tapped gently to mix and incubated on ice for 1 minute. The cell suspension was transferred to a 0.2cm cuvette and the cuvette placed in the Gene Pulser apparatus (BioRad, California, USA). The electrophoresis

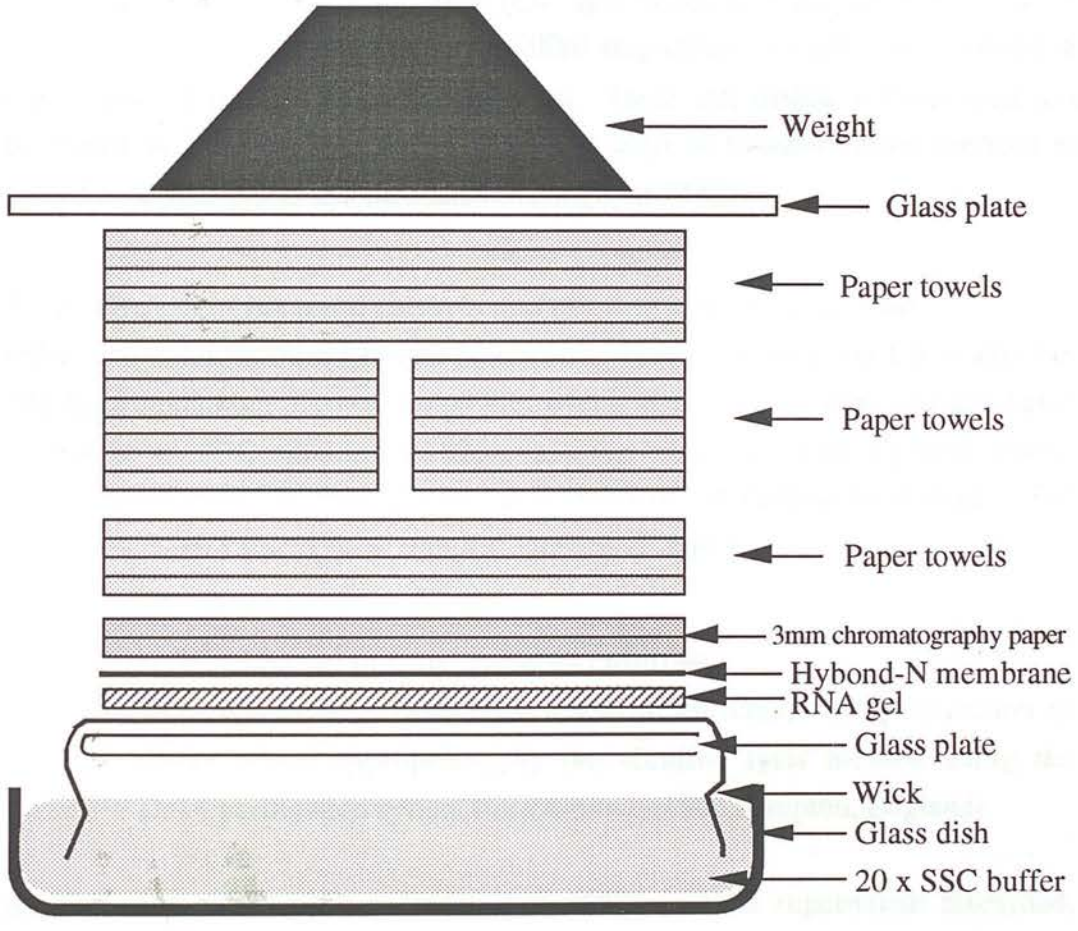


Figure 3.4. Schematic representation of the apparatus used to transfer RNA from a denaturing gel onto Hybond N membrane by capillary action.

reaction was activated (2.5μF, 200Ω, 2.5V) and maintained for 15 seconds. After this time, the cuvette was removed from the machine and 800μl SOC medium quickly added and the cells resuspended with a pipette. The SOC/cell mix was transferred to a 12 ml tube and placed in an orbital shaker at 37°C, 225 rpm for 1 hour. During this time, Luria Bertani (LB) agar plates were prepared. LB agar (Bio 101 Inc., Vista, CA, USA) containing 200μl ampicillan (50μg/ml) was poured into the bottom of petri dishes and left to set. Once set, dishes were moved to an incubator at 37°C to dry. 100μl, 50μl and 25μl of transformation reaction was spread onto agar plates and incubated overnight at 37°C.

3.9.2. Growth of bacterial cultures and glycerol stock preparation

Individual colonies from LB agar plates were transferred to 10 ml LB broth (Bio 101 Inc.) containing 50μg/ml ampicillin with a sterile plastic loop and incubated overnight at 37°C, shaking at 225revs. The next day, two glycerol stocks consisting of 600μl of 50% glycerol and 300μl of LB culture were made. The resultant glycerol stocks were stored at -20°C and -70°C.

3.9.3. Plasmid preparation from bacterial cultures

Plasmid DNA was isolated from 10ml bacterial cultures, after preparation of glycerol stocks where appropriate, by the alkaline lysis method using the Wizard™ DNA purification system from Promega (Southampton, England).

Briefly, suspensions were centrifuged at 1600g and the supernatant discarded, cells resuspended in buffer containing 50mM Tris/HCl, pH7.5, 10mM EDTA and 100μg/ml RNase and lysed with an equal volume of 0.2M NaOH and 1% SDS. The suspension was neutralised with 1.32M potassium acetate and centrifuged for 5min at 12,000g resulting in sedimentation of bacterial genomic DNA. The supernatant (approximately 600μl) was removed, mixed with 1ml DNA purification resin and passed down a miniprep column which retarded only the plasmid DNA. The column was washed with an ethanol based solution containing 0.2M NaCl, 20mM Tris/HCl, pH7.5 and 5mM EDTA. 50μl double distilled water heated to 65°C was added to the column and incubated at room temperature for 1min. DNA was eluted from the column by centrifugation at 12,000g for 1 minute

and a further 50µl double-distilled water added and the process repeated to give a final volume of 100µl.

3.9.4. Plasmid DNA quality and concentration

Purity of plasmid DNA was determined by analysis on a 0.8% agarose minigel. This was prepared using Seakem agarose dissolved in 1x TBE buffer (10X TBE consists of 0.089MTris base, 0.089M boric acid, 10mM EDTA; all from Sigma). The agarose was melted and approximately 200µg/ml ethidium bromide was added for visualisation of DNA. The gel was poured into a 7cm by 10cm gel tray containing an 8 well comb and submerged in 1 x TBE buffer in an electrophoresis submarine cell (Hoefer, Newcastle, England). Plasmid DNA (1µl) was run in a sample solution containing 1µl 'orange juice' (consisting of 0.25% (w/v) orange G, 15% (w/v) ficoll and 0.5M EDTA at pH7.0) and 8µl water. Samples were separated in parallel with pGem DNA markers (range 36-2,645bps, Promega) by electrophoresis at 100V for 1-2h in 1 x TBE, viewed under UV light and photographed. Pure plasmid DNA appeared as two visible 'bands' of greater than 2kb (depending on plasmid size); one for the circular DNA and the other representing supercoiled DNA which migrates more rapidly through the gel due to its compact form. Approximate plasmid DNA concentration was determined by comparing the sample with known concentrations, 100ng and 200 ng of plasmid DNA standards (pBR322, Promega) also run on the gel.

3.10. Preparation of radiolabelled probes for Northern blot analysis.

3.10.1. Preparation of plasmids for labelling

Plasmid DNA prepared as described previously (see section 3.9) was linearised in a reaction containing 1µg DNA, 10U restriction enzyme (SP64 and SP65 utilise Hind III ; Boehringer Mannheim, Lewes, East Sussex), reaction buffer B (contains 10mmol/l Tris/HCl, 5mmol/l $MgCl_2$, 100mmol/l NaCl and 1mmol/l ß-mercaptoethanol, pH8 ; Boehringer Mannheim) and RNase-free water to a volume of 30µl. The reaction was incubated at 37°C for 1-1.5h. Thereafter, 3µl of reaction mix was run on a minigel (0.8% agarose) and compared to an uncut sample of the same plasmid run in a parallel lane to test efficiency of digestion. Digested DNA was then extracted once with Tris-buffered phenol:chloroform (CAMLAB,

Cambridge, England) and precipitated with 1/10th volume 3M sodium acetate pH5.5 and 2 volumes absolute ethanol overnight at -20°C. Linearised DNA was pelleted, dried and resuspended in 10µl pure water.

3.10.2. Radioactive labelling of riboprobe for Northern analysis

Radioactive riboprobe was synthesised using linearised plasmid DNA (prepared as described in section 3.10.1) and SP6 RNA polymerase (Boehringer Mannheim). Linearised plasmid was incubated with ^{32}P UTP in a reaction mixture containing containing 10mM DTT, 20U RNase inhibitor, 1mM each rATP, rCTP and rGTP, transcription buffer (1 x transcription buffer consisiting of 40mM Tris/HCl, pH7.9, 6mM $MgCl_2$, 2mM spermidine and 10mM NaCl; all from Promega) and 50µCi ^{32}P-UTP (Amersham International). The reaction was catalysed by addition of 30U of the appropriate RNA polymerase (SP6, Boehringer Mannheim) and incubated at 37°C for approximately 1h. After this time 25µl phenol:chloroform was added and the tube vorted briefly and centrifuged at 13000rpm for 1 minute. The upper layer was decanted into a new tube and subjected to ethanol precipitation on dry ice for 30 minutes. The superntant was decanted and the pellet dissolved in 50µl RNase-free water and the activity of 2 x 1µl aliquots was determined by liquid scintillation spectroscopy. The radiolabelled riboprobe was then added to the hybridisation mixture (section 3.10.3)

3.10.3. Synthesis of 18S rRNA oligonucleotide

18S rRNA oligonucleotide was synthesised using phosphoramadite chemistry on a Model 381 DNA synthesiser (Applied Biosystems, Warrington, UK). Oligonucleotides were recovered into 1ml of pure concentrated ammonia, deprotected by incubating overnight at 65°C, recovered by two rounds of ethanol precipitation and resuspended in TE buffer (10mM Tris, 0.1mM EDTA). The concentration of the oligonucleotides were estimated by spectroscopy at 260nm where an optical density of 1.0 is equal to a concentration of 20µg/ml.

3.10.4. Radioladelling of oligonucleotide for Northern analysis

Oligonucleotides were labelled at the 5' end using polynucleotide kinase (PNK). DNA was incubated with [γ-^{32}P]ATP in a reaction mixture containing 50ng DNA, 30µCi [γ-^{32}P]ATP, 1 x kinase buffer (10 x buffer contains 0.5M Tris/HCl, 0.1M

$MgCl_2$, 50mM DTT, 1mM spermidine and 1mM EDTA) and 8U T4 PNK (Amersham). The reaction was allowed to continue at 37°C for 30 minutes. Unincorporated nucleotides were removed by passing the reaction mixture down a Clontech ChromaSpin +TE-10 spin column. The labelled oligonucleotide was then added directly to DNA hybridisation mix (section 3.11).

3.11. Northern blot analysis

3.11.1. Hybridisation of radiolabelled probe to membrane

Riboprobe Membranes were prehybridised at 65°C for 2-4 hours in buffer containing 0.2M sodium phosphate pH7.2, 1mM EDTA, 1% BSA, 7% SDS and 45% formamide. Radiolabelled probe was added to the hybridisation mix at a final concentration of 0.5 - 1 x 10^6 cpm/ml buffer. Hybridisation was allowed to continue for 24-48 hours at 65°C.

Oligonucleotide Probe Membranes were prehybridised at Tm of probe minus 10°C. Tm is the melting temperature of the probe dependent on the nucleotide content of the DNA and was calculated using the formula (4 x G+C) + (2 x A+T). Tm of 18S rRNA probe was 92°C. Prehybridisation buffer contained 0.05% w/v BSA, 0.05% w/v polyvinylpyrrolidine, 0.05% w/v ficoll, 0.1% w/v SDS, 0.1% w/v sodium pyrophosphate, 5 x SSC and 100µg/ml sonicated salmon sperm DNA (Sigma). Labelled probe was added to the mix and hybridisation allowed to proceed for 24-48 hours.

3.11.2. Post-hybridisation washes

Riboprobe Membranes were washed with buffer containing 40mM sodium phosphate pH7.2, 1mM EDTA and 1% SDS. Washes were at 65°C for 2 x 30 minutes.

Oligonucleotide Probe Membranes were washed with 4 x SSC at Tm minus 5°C for 2 x 30 minutes. For more stringent washes 1 x SSC, 0.5 x SSC or 0.1 x SSC were used.

3.11.3 Development of signal

After washing, membranes were air dried briefly and wrapped in clingfilm. The membrane was then exposed to X-ray film (XAR-5 or X-Omat S; Kodak) in cassettes with Dupont enhancing screens at -70°C. After a specific exposure time the

signal was developed using LX 24 developer and fixed using FX 40 fixative according to the suppliers recommendations (both Kodak).

3.12. Probe preparation for *in situ* hybridisation.

3.12.1. Template preparation

Linearised plasmid for use in in situ hybridisation was prepared as for Northern analysis and is described in section 3.10.1.

3.12.2. Preparation of radiolabelled riboprobe

Synthesis of riboprobes was carried out using 1µg linearised template in a reaction mix containing 10mM DTT, 20U RNase inhibitor, 1mM each rATP, rCTP and rGTP, transcription buffer (1 x transcription buffer consisiting of 40mM Tris/HCl, pH7.9, 6mM $MgCl_2$, 2mM spermidine and 10mM NaCl) and 50µCi ^{35}S-UTP. The reaction was catalysed by addition of 30U of the appropriate RNA polymerase (SP6) and incubated at 37°C for approximately 1.5h. The DNA template was then removed by digestion with 40U RNase-free DNase for 10min at 37°C. Enzymes and salts were removed by passing the radiolabelled sample through a Nu-Clean R50 disposable spin column (Kodak) according to the manufacturers instructions or by two cycles of phenol/chloroform extraction. RNA was precipitated with sodium acetate and ethanol overnight at -70°C and recovered by centrifugation as above.

Radiolabelled RNA was air dried, resuspended in 50µl RNase-free water and the activity of 2 x 1µl aliquots was determined by liquid scintillation spectroscopy. The average activity of the two samples was calculated and the volume of probe necessary to give 1 x 10^6 cpm was determined.

3.13. Radioactive *in situ* hybridisation.

3.13.1. Pretreatment of tissue and probe hydridisation.

Paraffin wax embedded tissue was cleared in histoclear for 15min and rehydrated in a series of alcohols with decreasing concentrations. Tissue was placed in 0.2N HCl for 20min followed by two 5min washes in distilled water. Sections were then

incubated in 2µg/ml proteinase K (Sigma) in buffer consisting of 20mM Tris/HCl pH7.4 and 50mM EDTA at 37°C for 20min followed by 0.2% glycine in water at 4°C for 10min. Sections were washed briefly in 0.1M triethanolamine (TEA; Sigma), pH8.0 and acetylated in 0.25% acetic anhydride (BDH Ltd) in 0.1M TEA, pH8.0 for 10min. Finally, sections were rinsed in distilled water and prehybridised in buffer containing 4 x STE (1 x STE contains 150mM NaCl, 2.5mM Tris and 0.25mM EDTA), 1 x Denhart's solution (50 x Denhart's contains 5g BSA, 5g polyvinylpyrrolidine and 5g ficoll in 500ml solution), 10mM DTT, 125µg/ml salmon sperm DNA, 125µg/ml yeast transfer RNA and 50% deionised formamide (all from Sigma) for 2-4h at 55°C. Excess prehybridisation buffer was removed from slides and replaced with probe at a concentration of $1x10^6$ cpm/40µl hybridisation buffer (as prehybridisation buffer plus 10% dextran sulphate)/slide. Incubation with probe was carried out beneath gel bond film coverslips (Flowgen Instruments Ltd.) in a humidified chamber at 55°C for 18hours.

3.13.2. Post hybridisation washes

After incubation, sections were washed twice with 4 x SSC over 15min to remove the coverslip and treated with RNase A (Sigma) at a concentration of 20µg/ml in 0.5M NaCl, 0.1M Tris, pH8.0 and 1mM EDTA for 30min at 37°C. Sections were washed for 30 min in RNase buffer alone at 37°C, followed by two 30 min washes in 2 x SSC at room temperature and 45°C, with a final 30 min wash in 0.5 x SSC at room temperature.

3.13.3. Development of *in situ* hybridisation

After post hybridisation washing, sections were dehydrated through alcohols containing ammonium acetate and air dried. Slides were warmed to 45°C and dipped in prewarmed NTB2 emulsion (Kodak) at 45°C in the dark. Emulsion coated slides were stored in a humidified, lightproof box overnight before transfer to a polyacetyl black trough (lightproof; Lamb's laboratory supplies, London, England) with silica gel. and stored at 4°C until developed at times specified in the relevent chapters. Silver grains formed by reaction of the hybridised, radiolabelled probe with the emulsion were developed using Kodak D19 developer as follows. Slides were placed in developer cooled to 14°C for 4min, followed by a brief wash in distilled water and grains were fixed by a 5 min

incubation in Kodak unifix at 14°C. Sections were washed in water, stained with haematoxylin, dehydrated and mounted in Pertex.

Slides were analysed under dark field using either BIOMED or LABORLUX 12 microscopes (Leitz, Wetzlar, Germany) or the Zeiss Photomicroscope III (Zeiss, Hertfordshire, England) to visualise the silver grains indicating areas of hybridisation.

Chapter 4. Identification and maturation of AVP neurons in serum-free cell culture

4.1. Introduction

The role of the hypothalamic neuropeptides, CRH and AVP in the release of ACTH from the fetal pituitary gland has been extensively demonstrated both *in vivo* (Brooks *et al.*, 1987; Apostolakis *et al.*, 1991; Brooks and White, 1990) and *in vitro* (Durand *et al.*, 1986; Brooks *et al.*, 1987; Lü *et al.*, 1994). However, the factors controlling the development of these neuropeptide neurons within the hypothalamus has been less widely studied. Recently, it has been suggested that growth factors play a role as putative neurotrophic factors to neurons of the central nervous system. Growth factors including basic fibroblast growth factor, nerve growth factor and brain derived neurotrophic factor have been shown to enhance survival and neurite outgrowth from CNS neurons (Walicke, Cowan, Ueno, Baird and Guillemin, 1986; Hofer and Barde, 1988; Alderson, Alterman, Barde and Lindsay, 1990; Arimatsu and Miyamoto, 1991). Insulin-like growth factor-1 has been implicated as a putative neurotrophic factor for fetal rat hypothalamic neurons (Torres-Aleman, Naftolin and Robbins, 1989; 1990). Addition of IGF-1 to primary hypothalamic cultures results in a significant dose-dependant increase in neuronal survival which is accompanied by increased levels of protein kinase C, which has previously been shown to increase in neuronal culture as a function of time and in parallel with increased neurite outgrowth and synaptogenesis (Girard, Wood, Freschi and Kuo, 1988).

The complex interactions of the hypothalamus make it difficult to study constituent neuronal populations in the intact animal. The development of a serum-free cell culture system for maintenance of rat hypothalamic neurons (Clarke and Gillies, 1988) provides a valuable tool for the investigation of hypothalamic neurons in a defined environment. In this way neurons of the hypothalamus can be studies in a defined situation, free from the complex interactions of the developing brain and the feedback influences of the HPA axis. AVP neurons *in vitro* respond to potassium depolarisation in a Ca^{2+} -dependant fashion with release of peptide and this response can be repeatedly evoked over a period of weeks. Stimulated release of AVP in response to potassium can be maintained for at least 4 hours, illustrating that cultured neurons are capable of synthesising mature peptide. In addition, secretion

can be inhibited by pretreatment with glucocorticoid demonstrating that the neurons maintain the ability to respond to physiological stimuli (Clarke and Gillies, 1988).

The development of neuronal systems is presumed to be regulated by the availability of neurotrophic factors. However, for most neuronal populations, including the developing AVP neurons the identity of these factors is unknown. The aim of this study was to identify AVP neurons within an *in vitro* culture system and to chart their growth and development with the long term goal of identifying putative neurotrophic factors which may influence their maturation. Central to this project was the ability to identify AVP neurons in culture by immunocytochemistry in order that the effects of putative neurotrophic factors on neurite outgrowth and AVP cell number could be examined. The secretion of peptide into the culture medium in response to potassium-induced depolarisation is often used as a measure of functionality of peptidergic neurons (Clarke and Gillies, 1988; Clarke, Lowry and Gillies, 1987; Sarkar and Sakaguchi, 1990; Currie *et al.*, 1994) and was used in this study to illustrate the presence of viable AVP neurons within the culture system. The influence of IGF-1 on the functional maturation of the neurons was assessed by determining the effect of incubation of hypothalamic cultures with differing doses of IGF-1 on basal and potassium-induced AVP secretion.

4.2. Materials and methods

4.2.1. Animals

Adult Wistar rats were obtained from an 'in-house' colony at the Centre for Reproductive Biology, Edinburgh. The rats were maintained in a 12 hour light: dark cycle with lights on at 7am and were given access to food and water *ad libitum.* Rats were time-mated by monitoring intra-vaginal smears to determine the stage of the female reproductive cycle. Females were placed with males for 48 hours spanning the period of the next oestrus cycle. The day of oestrus was designated day 0 of pregnancy. Pregnant female rats were housed together until fetal hypothalami were collected at day 17.5 gestation (gestation =21-22 days).

4.2.2. Treatment of glassware.

All glassware for use in the cell culture procedure was chemically cleaned to remove any surface contamination. Glassware was steeped overnight in 2% 'Micro'

detergent (International Products Ltd, Chislehurtst, Kent). The next day, the glassware was rinsed and immersed in sterile tissue culture grade water (Sigma) for several hours followed by a wash in 1M hydrochloric acid (BDH Ltd.). Glassware was then washed sequentially in sterile water, absolute alcohol and sterile water again before autoclaving and storing until ready for use.

All glassware which was to come into contact with cell suspensions was siliconised before use to prevent cell adhesion. Glassware was briefly immersed in 'Sigmacote' (Sigma) and left to dry in a fume-hood. Upon drying, the glassware was rinsed in absolute alcohol and washed several times in sterile water before autoclaving.

Pasteur pipettes (John Poulten Ltd, Barking Essex) of varying diameters were required during the tissue dispersion. Pipettes were heated gently in a Bunsen flame until the tips were red hot. Varying the amount of heating produced pipettes of differing tip diameter.

4.2.3. Preparation of media for tissue dispersion and cell culture

Dulbecco's Modified Eagle's Medium with phenol red and without l-glutamine (DMEM), Dulbecco's Phosphate Buffered Saline (DPBS), Ham's F12 Medium (with phenol red and without l-glutamine), Earle's Balanced Salt Solution without phenol red (EBSS) and Hank's Balanced Salt Solution with calcium and magnesium and without calcium and magnesium (HBSS with Ca^{2+}/Mg^{2+} and HBSS without Ca^{2+}/Mg^{2+}) were purchased from ICN Flow Ltd, Rickmansworth, Hertfordshire. Culture grade progesterone, oestradiol, Sodium selenite, transferrin, putrescine, gentamycin, insulin, triiodo-thyronine (T3), glucose, bovine serum albumin (BSA: fraction V), double processed tissue culture water, HEPES (1M stock) and deoxyribonuclease Type 1 (DNase) were purchased from Sigma Chemicals Co. Ltd., Poole, Dorset. Penicillin/Streptomycin (Penstrep, 5000IU/ml and 500µg/ml respectively) and glutamine were obtained from ICN Flow Ltd. Dispase (cell culture grade Neutral Protease) was obtained from BCL Ltd., London, UK. Insulin-like Growth Factor -1 was purchased from Peninsula Laboratories Europe Ltd., Merseyside, UK.

Media for tissue dispersion

Media for tissue dispersion were prepared one day in advance, filter-sterilised and stored at 4°C overnight. On the morning of dispersion, enzymes were added and the media were warmed to 37°C.

Collection buffer consisted of 100ml HBSS without Ca^{2+}/Mg^{2+} containing 500µl 20% BSA in water, 2ml 1M HEPES and 2ml Penstrep.

Dispersion medium consisted of collection buffer containing an additional 1.5ml 20% BSA. 20mg Dispase and 25mg DNase per 100ml were added on the day of use.

DNase medium was made according to the recipe for collection buffer, except that the HBSS contained Ca^{2+}/Mg^{2+}. 50mg DNase per 100ml was added to the medium on the day of use.

Stock solutions and media for cell culture

Stock solutions for preparation of cell culture media were prepared under sterile conditions and stored as stock solutions at 4°C or as frozen concentrates at -20°C until required. Stock solutions were replaced regularly to ensure freshness and sterility.

Triiodo-thyronine (T3) 5mg T3 were dissolved in 0.1M sterile sodium hydroxide solution to a concentration of 0.65mg/ml ($1x10^{-3}$M). This solution was further diluted in tissue culture grade water to a final concentration of $1x10^{-6}$M. Triiodo-thyronine solution was freshly prepared on each occasion.

Insulin stock 20mg of insulin were dissolved in 0.5ml 10%v/v acetic acid solution to a concentration of 10mg/ml. This solution was further diluted in tissue culture grade water to a final concentration of 5mg/ml, filter sterilised and stored at 4°C for up to one month.

Oestradiol -17β 3.67ml of sterile absolute ethanol was added to 1mg powdered oestradiol-17β, giving a $1x10^{-3}$M solution. This was further diluted to a concentration of $1x10^{-6}$M and 50µl of this solution was added to 50ml sterile EBSS, giving a final stock concentration of $1x10^{-9}$M. The stock solution was stored in a glass bottle at 4°C for up to 3 months.

Progesterone 1.59ml of sterile absolute ethanol was added to 1mg powdered progesterone, giving a final concentration of $2x10^{-3}$M. 500µl of this solution was

added to 50ml sterile EBSS giving a final stock concentration of $2x10^{-5}M$. The stock solution was stored and replaced as for the oestradiol solution.

Arachidonic acid 500mg arachidonic acid was diluted in absolute ethanol to a concentration of 100mg/ml. This solution was further diluted in DMEM/Hams F12 (1:1 v/v) to a concentration of 100µg/ml. Fatty acid free BSA (Sigma) was added to the stock solution to a final concentration of 2.5mg/100µg arachidonic acid. The stock solution was stored in siliconised glass vials at -20°C.

Docosahexaenoic acid 500mg docosahexaenoic acid was diluted in absolute ethanol and subsequently in DMEM/Ham's F12 as for arachidonic acid, to yield a stock solution of 100µg/ml. Fatty acid free BSA was added as described for arachidonic acid and the solution stored at -20°C in siliconised glass vials.

Combined Supplement for SFCM SFCM stock supplement consisted of 20 ml glutamine solution (29.2mg/ml, 0.2M), 20ml transferrin solution (10mg/ml), 2ml putrescine solution (16.11mg/ml, 0.1M) and 20µl sodium selenite (0.52mg/ml, $3x10^{-3}M$). The supplement was filter-sterilised and frozen in aliquots at -20°C for up to one month.

Serum-Free Culture Medium 500ml serum-free culture medium (SFCM) consisted of 250ml Ham's F12 medium and 250ml DMEM containing 500µl T3 stock solution ($1x10^{-6}M$), 500µl insulin solution (5mg/ml), 500µl oestradiol solution ($1x10^{-9}M$), 500µl progesterone solution ($2x10^{-5}M$), 500µl gentamycin (50mg/ml), 10ml penstrep solution, 5ml arachidonic acid (1µg/ml), 2.5ml docosahexaenoic acid (0.5µg/ml) and 10.5 ml of combined supplement. The serum-free medium was then filter-sterilised and stored at 4°C for up to 7 days. The final composition of serum-free culture medium is described in Table 4.1.

Insulin-like Growth Factor-1 100µg IGF-1 was diluted in 10mM hydrochloric acid to give a concentration of 10µg/ml. BSA was added at a concentration of 1mg/ml and the stock aliquoted and stored at -70°C until required. On the day of use the stock solution was further diluted in SFCM to the desired concentration.

4.2.4. Coating of culture dishes

On the morning of dispersion, a solution of poly-l-lysine (10µg/ml ; Sigma, MWt. 70,000-150,000kD) was prepared in sterile tissue culture grade water. 1ml of poly-l-lysine solution was added to each well of a 35 mm gamma-irradiated Nunc culture dish (Gibco Ltd., Paisley, Scotland) or to each well of a 22mm culture dish containing a glass coverslip cleaned as described above for 30 minutes. After this

Table 4.1. Composition of Serum-Free Culture Medium

Component	Final concentration
DMEM/Ham's F12	50% : 50%
Penicillin	100IU/ml
Streptomycin	100µg/ml
Gentamycin	50µg/ml
Glutamine	1.5µg/ml
Insulin	5µg/ml
Transferrin	100µg/ml
Putrescine	100µmol/l
Selenium	30nmol/l
Triiodo-thyronine	1nmol/l
Progesterone	20nmol/l
Oestradiol 17β	1pmol/l
Arachidonic acid	1µg/ml
Docosahexaenoic acid	0.5µg/ml

time, the poly-l-lysine was aspirated and the wells were washed twice in sterile water. Subsequently. 1ml of SFCM containing 20% fetal calf serum (ICN Flow Ltd.) was added to each well. Culture plates were returned to a humidified incubator at 37°C, 95% air : 5% CO_2 atmosphere until ready for use following the dispersion.

4.2.5. Collection of fetal hypothalami and tissue dispersion

On day 17.5 gestation, pregnant females were sacrificed by CO_2 asphyxiation followed by cervical dislocation. Fetuses were removed aseptically, decapitated and placed into ice cold sterile saline. Fetal hypothalami were dissected and placed into 20ml sterile collection buffer.

Hypothalami were washed by gentle rotation, centrifuged at 300rpm for 1 minute, and resuspended in fresh collection buffer prewarmed to 37°C. The washing procedure was repeated 3 times. Following the washes, the tissue was resuspended in 20ml dispersion medium and the suspension transferred to a 50ml Bellco stirrer flask (Arnold Horwell Ltd., Werst Hampstead, London). The stirrer flask was placed in an incubator at 37°C and the suspension was stirred at 75rpm. After 30 minutes, the stirrer flask was removed from the incubator and large pieces of tissue were allowed to settle. The supernatant was aspirated to a sterile universal, hereafter called harvest 1 and returned to the incubator for short-term storage. A further 20ml of dispersion medium was added to the tissue in the stirrer flask and the tissue was triturated using two sterile, siliconised pipettes of decreasing diameter (approximately 1mm and 0.5mm). The triturated cell suspension was returned to the incubator and allowed to stir for a further 30 minutes. The supernatant was then aspirated and stored in a sterile universal, hereafter called harvest 2. A little dispersion medium was left in the stirrer flask and the remaining tissue was further triturated with pipettes of approximately 0.5mm and 0.2mm diameter. The resultant cell suspension was added to harvest 2. Harvests 1 and 2 were centrifuged for 10 minutes at 1000rpm for 10 minutes and the supernatants discarded. The pellets were resuspended and pooled in 20ml of DNase medium and the suspension returned to the incubator for 20 minutes, with occasional end over end rotation. The suspension was then centrifuged at 1000 rpm for 10 minutes and the supernatant discarded. The cells were resuspended in 5ml of collection buffer and the suspension was gently pipetted on top of 20ml collection buffer containing 4% BSA. The layered suspension was centrifuged at 1000rpm for 10 minutes to remove debris. The

pelleted cells were resuspended in 2-3ml of SFCM containing 20% fetal calf serum. The total cell number and viability were assessed using a haemocytometer and trypan blue exclusion. The suspension was diluted to $2.5x10^6$ cells/ml and 1ml of suspension was added to each 35mm culture well for stimulation experiments. For immunocytochemistry experiments, the suspension was diluted to $9.5x10^5$ cells/ml to give the same cell density ($2.5x10^5$cells/cm^2) in a smaller culture dish.

4.2.6. Maintenance of cell cultures

After 24 hours of culture, the medium was changed to SFCM containing 5µM cytosine β-D arabinofuranoside, an anti-mitotic agent included to aid the reduction in the number of dividing glial cells in the culture. After a further 24 hours, the medium was changed to SFCM alone or SFCM containing IGF-1 at either 1ng/ml, 10ng/ml or 100ng/ml. Thereafter, the media was replaced every 3 days or more often as required with fresh SFCM. For cells cultured in the presence of IGF, IGF was added to the SFCM at the same concentration at each media change. In some cultures insulin was omitted from the SFCM thereby providing two groups of cells, those cultured with IGF-1 in the presence of insulin and those cultured with IGF-1 in the absence of insulin.

4.2.7. Peptide release media

Basal Medium 100ml of basal release media consisted of EBSS supplemented with 1ml fresh ascorbic acid solution (Sigma, 3mg/ml in tissue culture grade water), 1ml sterile bacitracin solution (Sigma, 3mg/ml in tissue culture grade water), 1ml HEPES (1M stock), 2ml penstrep, 800µl aprotinin solution ('Trasylol', Bayer UK Ltd., Newbury, 10,000KIU/ml), 100mg D-glucose (Sigma) and 500µl 20% BSA in water. The medium was filter sterilised and stored at 4°C for up to 7 days.

56mM Potassium-containing medium EBSS with raised potassium levels and isotonically reduced sodium levels was made in the laboratory. The components of the basic salt solution were accurately weighed and all, except for calcium chloride were dissolved in 400ml tissue culture grade water. The calcium chloride was dissolved separately in 100ml of sterile water and was added to the 400ml salt solution slowly, with vigorous mixing of the salt solution to prevent precipitation. The solution was filter sterilised and stored at room temperature. The medium was supplemented prior to use as described above for the basal release medium.

4.2.8. Peptide release protocol

SFCM was aspirated from the cultures and cells were equilibrated for two 5 minute periods in 1ml prewarmed basal release media. After the equilibration period, the medium was aspirated and replaced with 700µl fresh basal release medium and the cells returned to the incubator for 3 hours. The basal release medium was then collected as two 350µl aliquots and stored at -20°C. The basal release medium was replaced with 700µl of 56mM potassium-containing medium and the cells returned to the incubator for a further 3 hour period. At the end of this time, the medium was collected and stored as before and the cells were washed three times with 1ml of prewarmed DMEM. Finally, 1ml of serum-free culture medium was added to each well and the cells returned to the incubator.

To assess the ability of the cultured neurons to produce AVP throughout the duration of the culture period, cultures were subjected to repeated stimulations performed on day 7, 10, 13, 16 and 20 of cell culture.

4.2.8. AVP radioimmunoassay

Buffer Assay buffer consisted of 0.05M phosphate buffered saline containing EDTA (1mmol/l), 0.1%NaN_3 and 0.1% BSA.

Antibody AVP antiserum (NK-2) was kindly supplied by Dr. N. J. Kasting, Department of Medical Physiology, University of British Columbia, Vancouver, Canada. NK-2 antibody was raised in a rabbit and shows minimal cross-reactivity with oxytocin (<0.2%, Kasting, Carr, Martin, Blume and Bergland , 1983). NK-2 antibody was added to the assay at a dilution of 1:100,000.

Iodination 5µg AVP was iodinated by the chloramine-T method and purified by passing through a sep-pak column as described in section 3.4.1.

Standards and Quality Controls 1mg AVP peptide (Cambridge Research Biochemicals, Northwich, Cheshire) was dissolved in 0.1M acetic acid to a concentration of 500µg/ml. Standards in the range 500pg/ml to 5pg/ml were prepared as serial dilutions in assay buffer. Quality controls consisted of three different concentrations of AVP in EBSS release medium.

Separation Bound and free ^{125}I-AVP were separated using magnetic donkey anti-rabbit IgG particles as described in section 3.4.3.

Method 100µl of standard, sample or quality control was incubated overnight at 4°C with 200µl assay buffer and 100µl NK-2 antiserum. The next day, 100µl

radiolabelled AVP (15000cpm/100µl) was added to each tube, the tubes were vortexed and incubated overnight at 4°C. The following day, bound peptide was separated by magnetic separation as described previously and the pellet counted for 60 seconds in a gamma counter.

Sensitivity The assay had a lower limit of detection of 1.75pg/ml and had intra- and inter-assay coefficients of variation of 16% and 21.8% respectively.

4.2.9. Immunocytochemistry

A range of AVP antibodies were screened using the avidin-biotin horse radish peroxidase (AB-HRP) immunocytochemistry protocol described in section 3.7. Cells to be utilised for immunocytochemistry were grown on coverslips as described above. The immunocytochemical procedure was carried out in the culture well. The coverslip was removed at the end of the staining procedure and mounted onto a glass microscope slide for viewing.

Alkaline phosphatase staining method

Antibodies raised against the neurophysin molecule were examined utilising an alkaline phosphatase detection system which utilises nitro-blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and levamisole as substrates. This method follows the same basic protocol as the avidin-biotin HRP method except that the avidin-biotin complex is linked to alkaline phosphatase (AB-AP) rather than HRP. The method is the same as that described in section 3.7 until the point of incubation with the avidin-biotin complex. Following the 30 minute incubation with the AB-AP complex, the cells were washed in buffer containing 100mM Tris pH 9.5, 100mM NaCl and 50mM $MgCl_2$ for 5 minutes. Cells were then incubated in the colour development solution containing 0.3mg/ml NBT, 0.175mg/ml BCIP and 0.24mg/ml levamisole (all from Sigma). Cells were incubated with the colour development solution in a dark humidified chamber at room temperature until staining was optimal.

Antibodies

Anti-AVP antisera used in this study came from a variety of sources. One antibody was obtained commercially (Arg^8- AVP: Biogenesis Ltd., Bournemouth, England). A range of polyclonal antibodies raised in both rabbit (TG1 and TG2) and sheep (S277, S278 and S279) were the gift of Dr. Alberto Smith, Department of Child Life and Health, The University of Edinburgh. Two further polyclonal antibodies (4S41

and 4S12) raised in sheep were the gift of Dr R.J Bicknell, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge. Monoclonal antisera raised against the neurophysin carrier molecule (PS41 and PS45), which had previously identified AVP-neurophysin containing cells in tissue sections and hypothalamic slice culture (Whitnall, Key, Ben-Barak, Ozato and Gainer , 1985, Gainer and Wray , 1992), were the kind gift of Dr.S.Wray, National Institute of Health, Bethesda, Maryland. Staining for Neuron-specific enolase (NSE: Cambridge Research Biochemicals, Northwich, Cheshire) which stains all neurons of the central and peripheral nervous system was also performed.

In order to assess the ability of the antibodies screened in cell culture to perform in the immunocytochemical protocol, the antisera were tested on paraffin-wax embedded sections of rat hypothalamus prepared as described in section 3.6. Specificity of the antisera was assessed by incubation of the antibody with specific antigen prior to use in the immunohistochemical procedure. Primary antibody was diluted in blocking buffer as described previously to which 5μM AVP peptide was added and the antigen-antibody mixture left to incubate overnight at 4°C. Following preabsorption of the antibody with AVP peptide, the preabsorbed antibody was treated as a primary antibody in the protocol.

4.2.10. Data analysis

AVP concentration in the culture supernatants was expressed as pg/well. Basal and potassium-induced AVP secretion from cells at different days in culture were analysed by Analysis of Variance (ANOVA). AVP secretion on different culture days from cells plated at different densities or in the presence of varying concentrations of IGF was analysed by two-way ANOVA. Where indicated in the text, difference between basal and stimulated means were analysed by Students t-test. All data are expressed as mean ± standard error of mean (SEM) and values of $p \leq 0.05$ were considered to be significant.

4.3. Results

4.3.1. Maintenance of hypothalamic neurons in culture

Dispersed rat hypothalamic neurons plated at a density of $2.5x10^5$cells/cm^2 in 35mm cell culture dishes were maintained in culture for 20 days. The dispersion procedure yielded a cell suspension with 95.14 ± 0.43% viability and each hypothalami contributed on average $1.03x10^6$ cells.

Figure 4.1 shows the effects of potassium-induced depolarisation of neuronal cultures plated at $2.5x10^5$cells/cm^2 in a 35mm culture dish on AVP secretion over the 20 day culture period. Each value represents the mean of 5 cultures from different dispersions and the value for each dispersion is the mean of 3 separate culture wells. Throughout the 20 day duration of the culture, the AVP neurons are secreting a basal level of AVP into the medium which is not significantly altered throughout the duration of the culture period. AVP secretion can be repeatedly stimulated by potassium-induced depolarisation throughout the culture period. Potassium-induced secretion of AVP is significantly increased ($p<0.05$) from basal levels on days 13, 16 and 20 *in vitro*.

The overall aim of the experiment was to identify AVP neurons from an early stage in culture and to influence the growth and development of these neurons. Initial studies were performed to determine the optimal density for visualisation of neurons in culture and to determine a density at which cells could be stained immunocytochemically whilst still at a density high enough to respond to potassium stimulation. Cells were plated at $2.5x10^5$, $1.5x10^5$ and $0.6x10^5$ cells/cm^2 in 22mm culture dishes and neuronal outgrowth and response to potassium-induced stimulation examined. Cells plated at $2.5x10^5$ cells/cm^2 respond to potassium-induced stimulation on day 13 and 20 of culture with significantly increased release of peptide whilst potassium treatment of cells at the two lower densities did not result in significant release of AVP (Figure 4.2). The level of both basal and stimulated AVP secretion is lower for the $2.5x10^5$ cells/cm^2 than for cells plated at the same density in Figure 4.1. The reduced level of secretion is likely to reflect the lower overall number of AVP neurons in the smaller dishes.

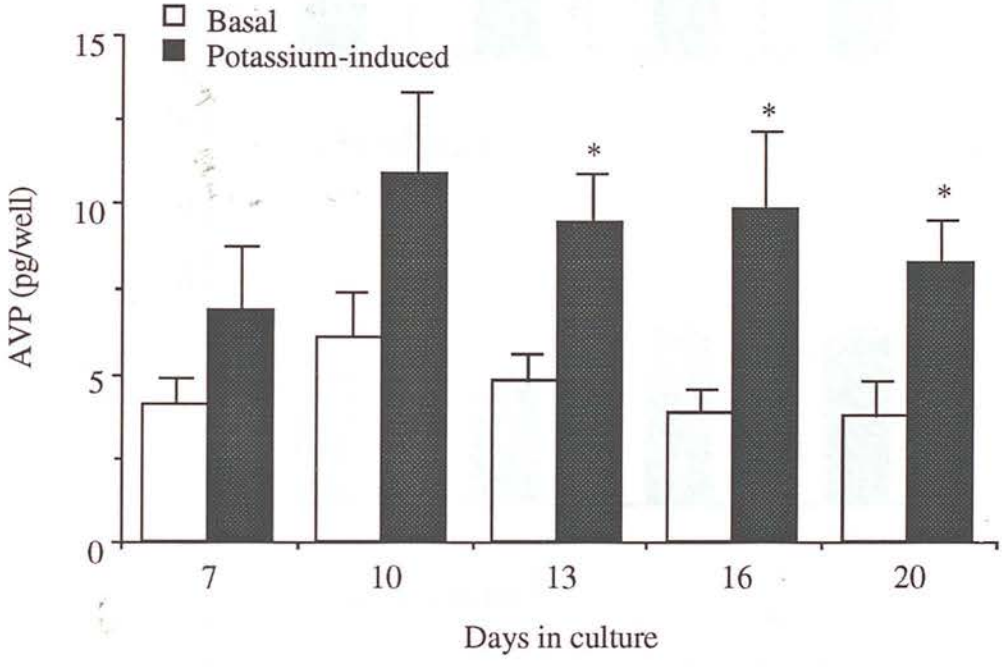


Figure 4.1. AVP secretion in day 17.5 gestation fetal rat hypothalamic cultures plated at $2.5x10^5$ cells/cm^2 in 35m culture dishes. Basal and potassium-induced AVP secretion was measured on day 7, 10, 13, 16 and 20 of culture. Data shown are the means of 5 separate dispersions where the value for each dispersion represents the mean of 3 culture wells, ± SEM. Significant differences between basal and potassium-induced AVP secretion are indicated by asterisks (* $p<0.05$; t-test).

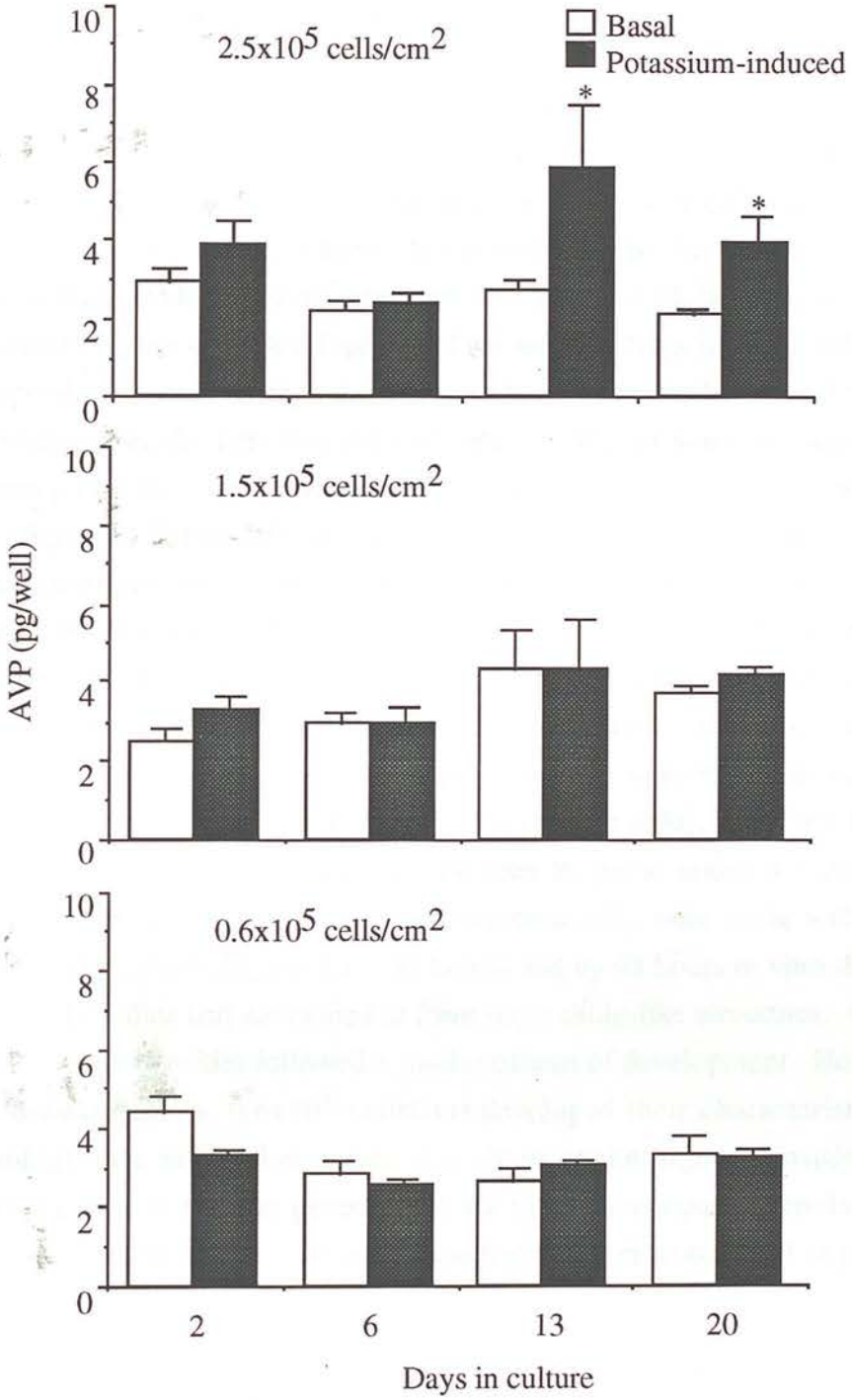


Figure 4.2. Basal and potassium-induced AVP secretion from day 17.5 gestation fetal hypothalamic cultures plated at $2.5x10^5$, $1.5x10^5$ and $0.6x10^5$ cells/cm^2 in 22mm culture dishes. Data shown represent the means of 10 or more wells ± SEM. Significant differences between basal and potassium-induced AVP secretion are indicated by asterisks (* $p<0.05$; t-test).

Visualisation of individual neurons was possible at $2.5x10^5$cells/cm^2 by phase-contrast microscopy. Cultures of hypothalamic neurons plated at this density on poly-l-lysine coated coverslips to be utilised for immunocytochemistry were examined frequently during the early stages of the culture period and were photographed under phase-contrast to obtain a profile of total neuronal growth of hypothalamic neurons *in vitro* during this period. The profile of neuronal growth of cells plated at $2.5x10^5$cells/cm^2 obtained over the first 48 hours of cell culture is illustrated in Figure 4.3. Development of the neurons from rounded cells following the dispersion procedure (Figure 4.3a) to cells of a classical neuronal morphology was evident over the first two days of culture. Whilst some neurons developed elaborate processes which extended in many directions others were bipolar. By 17 hours (Figure 4.3b) in culture most cells had extended neural processes. The neurons continued to extend neurites and began to form connections with other neurons. By the time of the first media change at 24 hours (Figure 4.3c), many neurons had attached firmly to the culture dish. Glial cells were also evident in the culture as flattened fibroblastic cells. By 48 hours *in vitro*, the dispersed hypothalamic neurons had a morphology typical of neuronal cells and the cells formed an extensive network of connections (Figure 4.3d). The cell body of the neuron is rich in cytoplasm and could be seen to 'glow' under the phase-contrast microscope. More connections between neuronal cells were made with increasing duration of the culture (Figure 4.3e ; 30 hours) and by 48 hours in vitro the processes between cell bodies had developed to form large cable-like structures. Cells plated at the two lower densities followed a similar pattern of development. However, cells at the lowest density, $0.6x10^5$ cells/cm^2 developed their characteristic neuronal morphology at a much slower rate than those at the higher densities (data not shown). In view of the data generated by the potassium-induced depolarisation and visualisation studies, all subsequent experiments were conducted at a density of $2.5x10^5$ cells/cm^2.

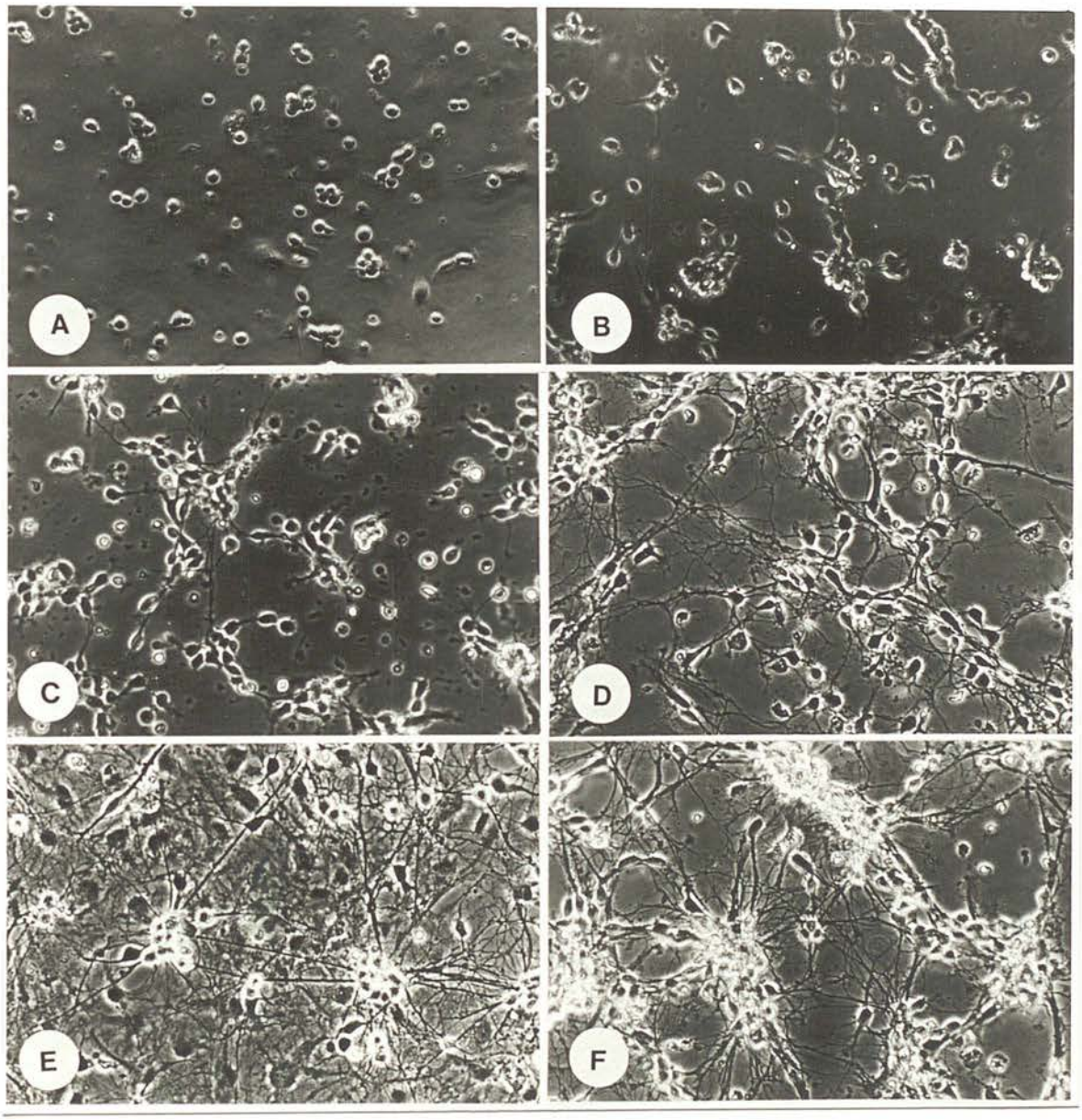


Figure 4.3. Phase-contrast microscopy of a representative hypothalamic culture illustrating neuronal growth over the first 72 hours of cell culture. Photographs represent A. 5 hours, B. 17 hours, C. 22 hours, D. 30 hours, E. 47 hours and F. 72 hours post-dispersion. All photographs were taken at x20 magnification.

4.3.2. Hypothalamic neurons cultured in the presence of IGF-1

Figure 4.4 shows AVP secretion from neurons cultured in insulin-containing and insulin-deficient SFCM. Cells cultured in insulin-containing SFCM secreted more AVP under basal conditions and responded significantly better to potassium-induced stimulation between days 7 and 13 of culture than cells cultured in the insulin-deficient SFCM. Neurons in insulin-deficient medium were able to compensate for the lack of insulin over time such that the insulin-deficient and insulin-containing cultures had similar responses to potassium-induced stimulation by day 20 of culture. Hypothalamic neurons cultured in insulin-containing SFCM containing IGF-1 at 100ng/ml, 10ng/ml and 1ng/ml are shown in Figure 4.5. Addition of IGF-1 does not have an additive effect when added to culture medium which contains insulin. Similarly, addition of IGF-1 to insulin-deficient SFCM did not significantly influence either basal or potassium-induced AVP secretion from cultured hypothalamic neurons when compared to SFCM alone control values (Figure 4.6). In both insulin-containing and insulin-deficient groups, basal secretion of AVP was not significantly altered over the duration of the culture period. Similarly, basal and potassium-stimulated levels of AVP secretion were not significantly different between groups at any day in culture.

4.3.3. Immunocytochemical identification of AVP neurons

Antibodies to be screened for staining of AVP neurons *in vitro* were first characterised using paraffin-embedded sections of rat hypothalamus. Staining of rat hypothalami with anti-NSE antisera resulted in intense staining of the neuronal fibres present in the hypothalamus, as shown in figure 4.7a. AVP antibodies screened displayed varying degrees of success. Figures 4.7c and 4.7e show representative sections of rat hypothalamus which stained successfully for AVP with anti-AVP antibodies raised in rabbit. Specificity of NSE and AVP staining in the hypothalamus was confirmed by the lack of staining upon omission of the primary antibody (Figure 4.7b) or preabsorption of AVP antisera with 5μM AVP (Figure 4.7d and 4.7f). Generally, antibodies raised in rabbit performed better than those raised in sheep (Figure 4.7g), which tended to have a higher level of non-specific staining as illustrated by the background staining present in the non-immune sheep serum negative control of Figure 4.7h. Antibodies which stained specifically for AVP in hypothalamic tissue sections were then screened for identification of AVP neurons in the culture system.

Figure 4.4. Basal and potassium-induced AVP secretion from hypothalamic cultures maintained in insulin-containing or insulin-deficient serum-free culture medium. Cells maintained in insulin-deficient SFCM tended to secrete less AVP than neurons which were maintained in insulin-containing SFCM. By day 16 of culture, there was no difference in secreted levels of AVP between the two groups. Data shown are the mean values of 4 culture wells ± SEM. Significant differences in basal or potAssium-induced AVP secretion from cultures maintained in insulin-containing and insulin-deficient SFCM are represented by asterisks (* , **, ***, $p<0.05$, $p<0.01$ and $p<0.001$ respectively).

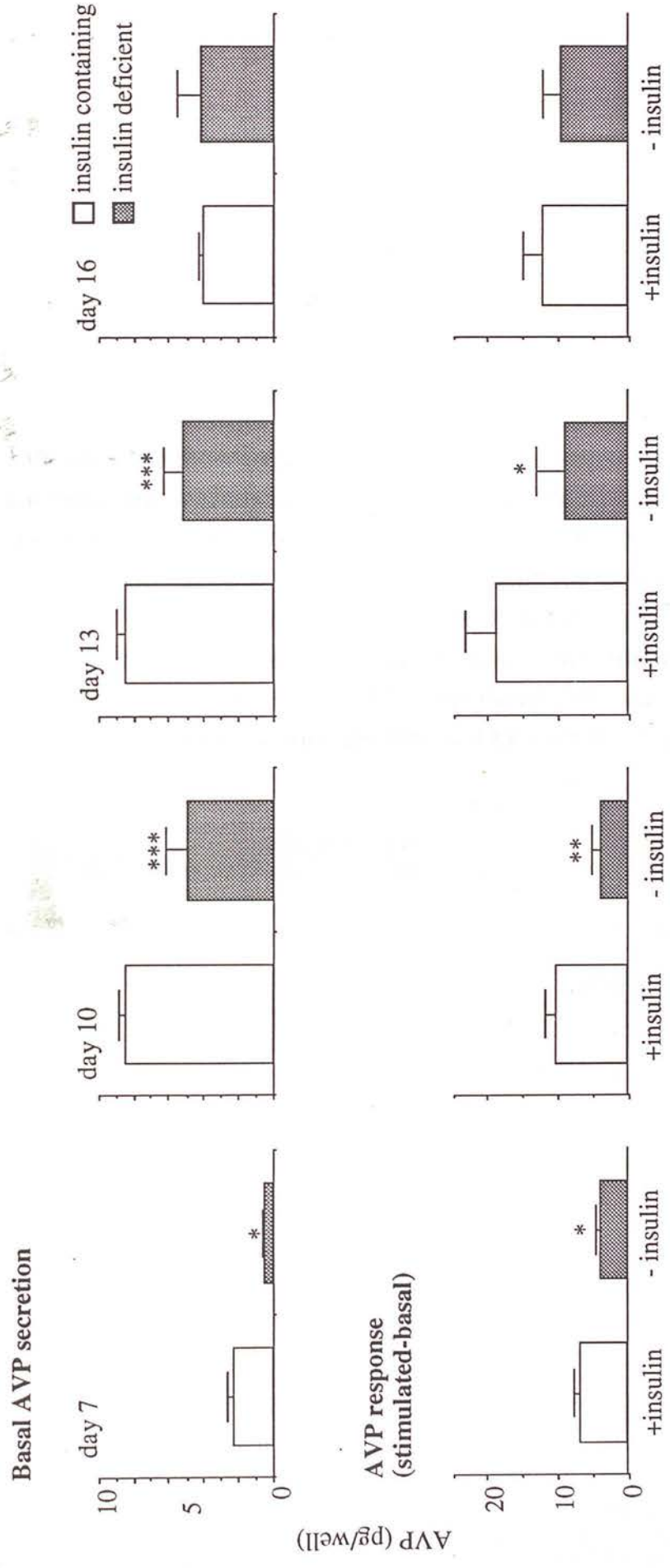

Basal AVP secretion
AVP response (stimulated-basal)
day 7
day 10
day 13
day 16
insulin containing
insulin deficient
AVP (pg/well)
10
5
0
20
10
0
*
**
***
+insulin
- insulin
Treatment

Figure 4.5. AVP secretion from hypothalamic neurons maintained in insulin-containing serum free culture medium alone or supplemented with IGF-1 at 1ng/ml, 10ng/ml or 100ng/ml. There were no significant effects of IGF-1 on basal or potassium-induced AVP secretion at any dose tested when compared to SFCM alone controls. Two-way ANOVA revealed that basal and potassium-induced secretion of AVP did not change significantly over the 20 day duration of the culture period. Data shown are the means of 9 culture wells ± SEM. Significant difference between basal and potassium-induced AVP secretion are indicated by asterisks, * ($p<0.05$), ** ($p<0.01$).

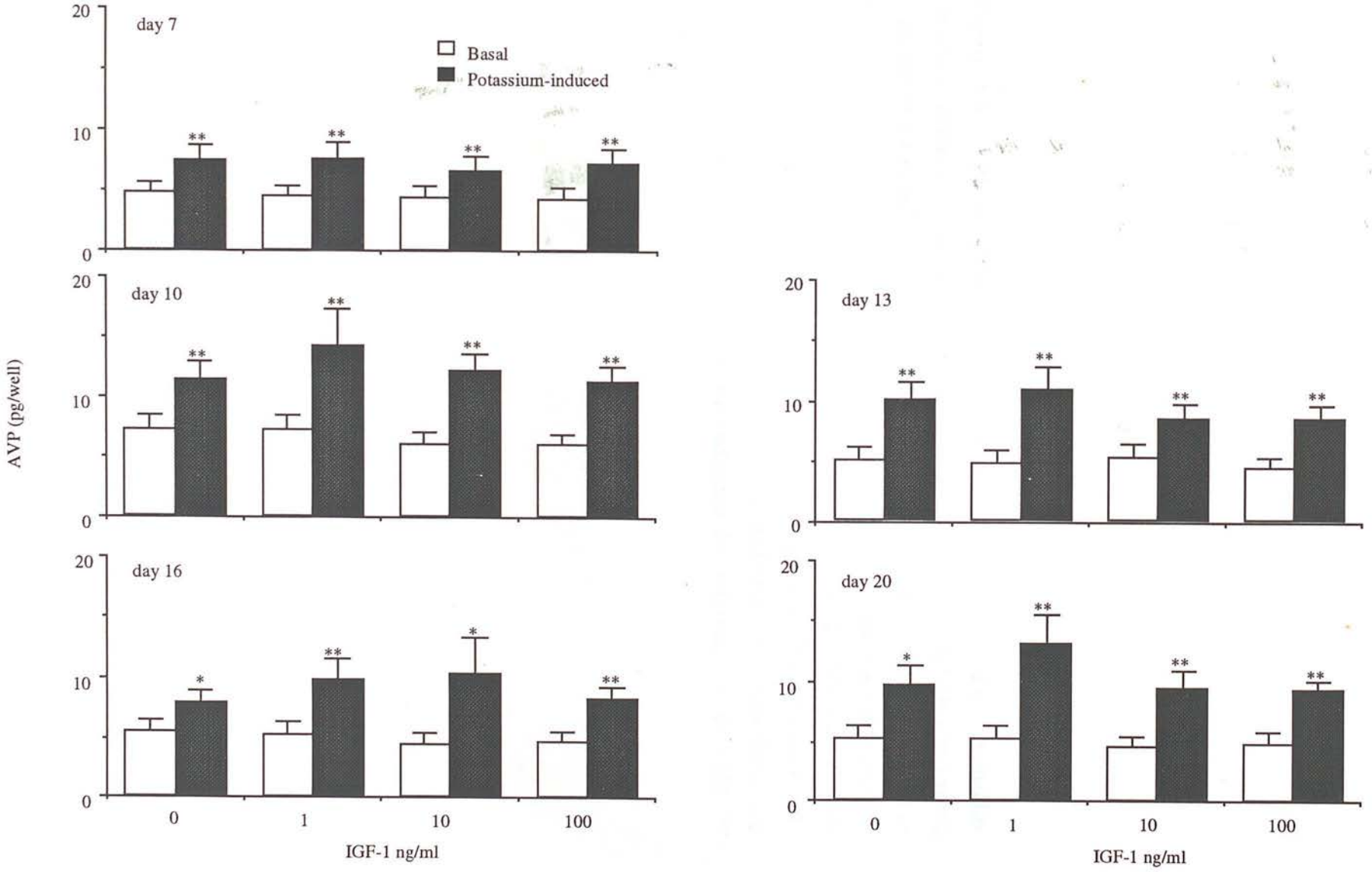
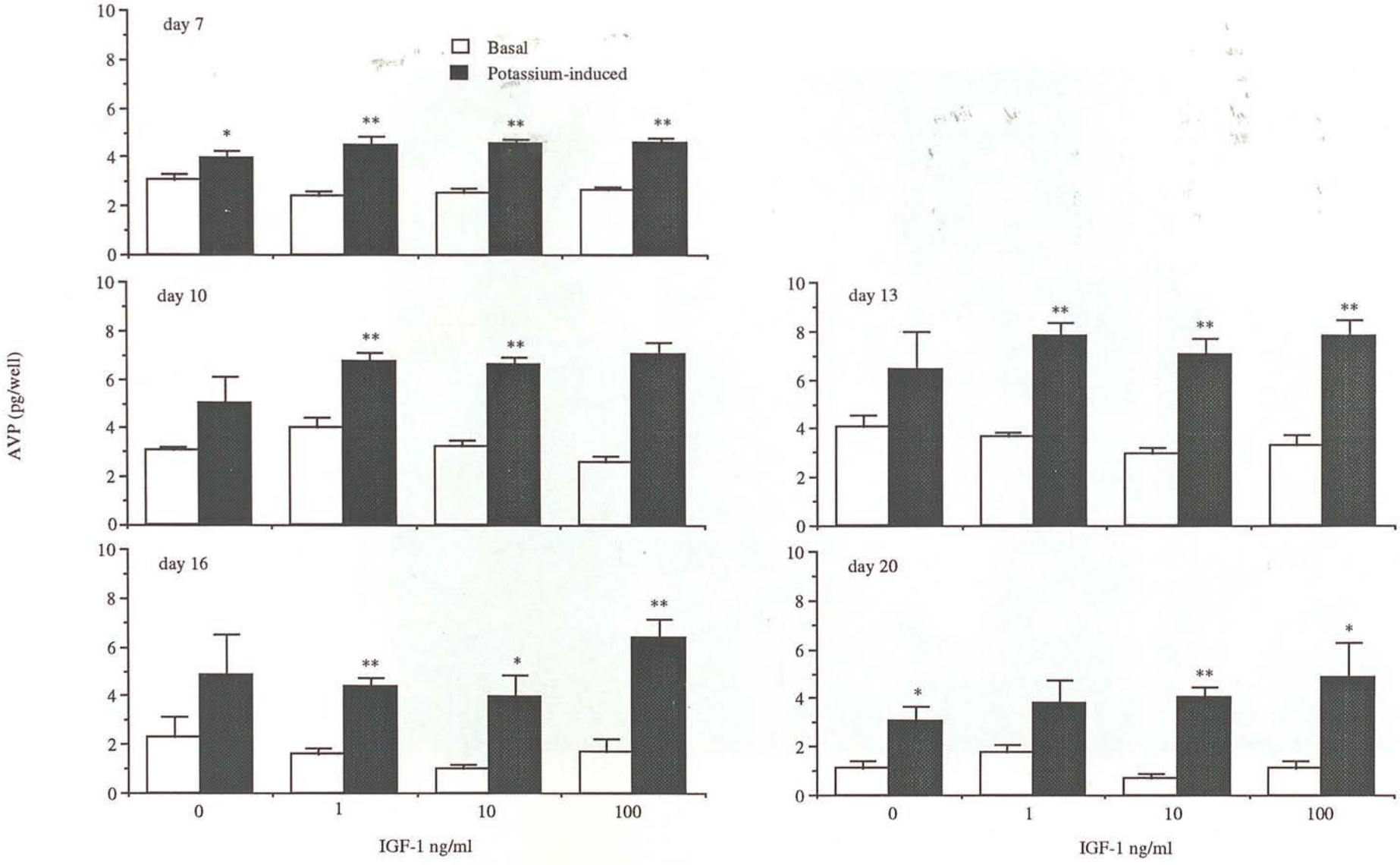

day 7
Basal
Potassium-induced
day 10
day 16
day 13
day 20
AVP (pg/well)
IGF-1 ng/ml
IGF-1 ng/ml
0
1
10
100
20
10
0
**
*

Figure 4.6. AVP secretion from hypothalamic neurons maintained in insulin-deficient serum free culture medium alone or supplemented with IGF-1 at 1ng/ml, 10ng/ml or 100ng/ml. Addition of IGF-1 to the SFCM had no significant effect on basal or potassium-induced AVP secretion at the doses tested when compared to SFCM alone controls. Basal and potassium-induced AVP secretion was not significantly altered over the duration of the culture period. Data shown are the means of 6 culture wells ± SEM. Significant difference between basal and potassium-induced AVP secretion are indicated by asterisks, * ($p<0.05$), ** ($p<0.01$).


day 7
Basal
Potassium-induced
day 10
day 13
day 16
day 20
AVP (pg/well)
IGF-1 ng/ml
IGF-1 ng/ml
0
1
10
100
0
2
4
6
8
10
*
**

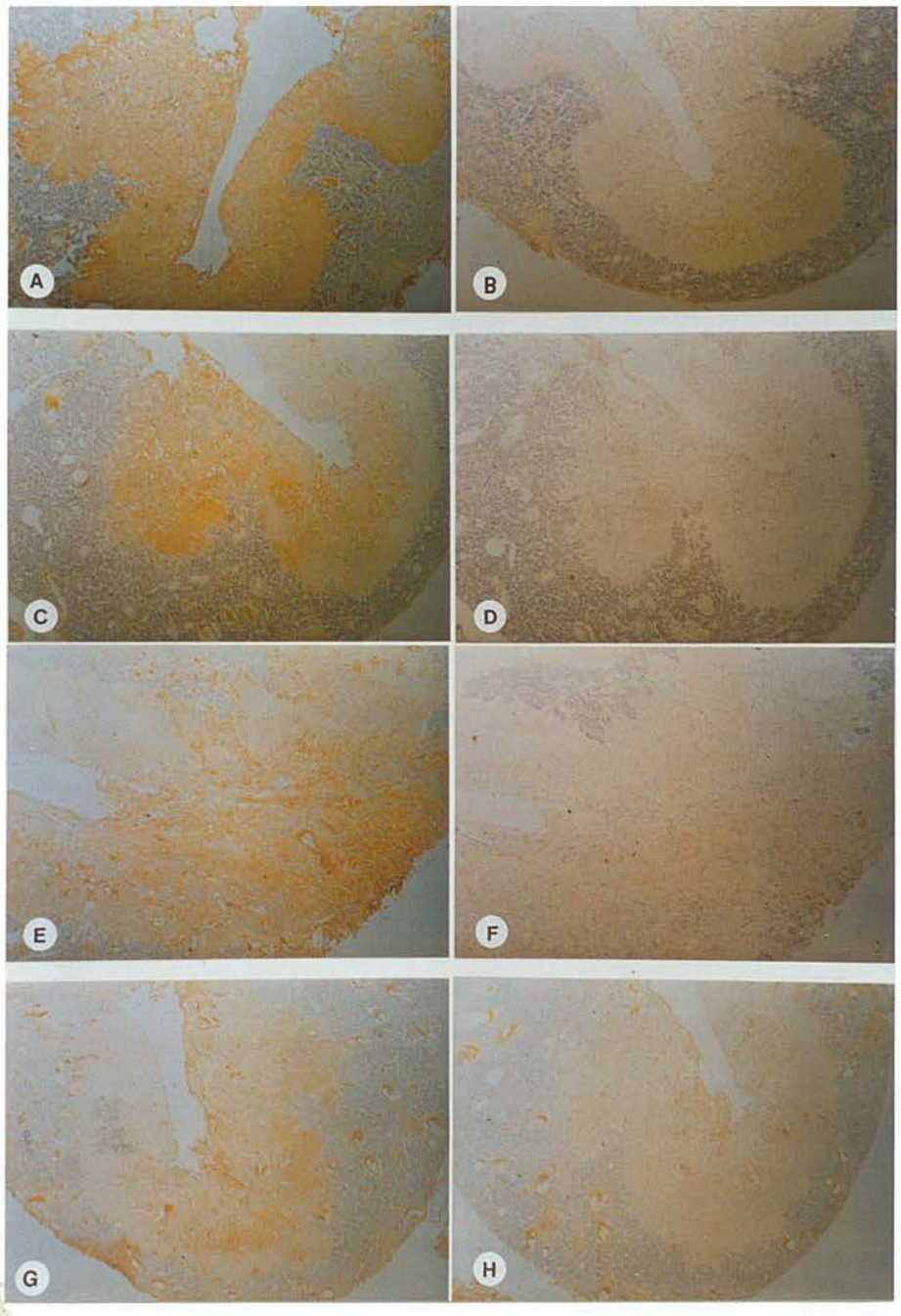


Figure 4.7. Immunohistochemical staining of rat hypothalamus with antibodies raised against NSE (A) and AVP (C, E and G). NSE antiserum resulted in strong staining of the neuronal fibres in the hypothalamus. Non-immune rabbit serum was used as a negative control for NSE (4.7B). AVP antibodies tested showed varying degrees of staining. Rabbit anti-AVP antibodies, TG2-4 (C) and TG1-10 (E) resulted in strong staining of AVP neuronal fibres. The specificity of staining was demonstrated by preabsorption of the antibody with 5μM AVP antigen (D, F). Anti-AVP antibody, S279 (G) raised in sheep also stained AVP fibres in the hypothalamus. Non-immune sheep serum was used as a negative control (H).

Hypothalamic cell cultures maintained for 20 days were successfully stained for NSE at the end of the culture period (Figure 4.8a). Staining for NSE was therefore used to validate many steps of the immunocytochemical protocol such as choice fixative and duration of fixation. Since the studies on AVP growth and maturation would involve fixation of cultures at many different stages which could then all be stained at the one time, cells were cultured to identify the best method of storage of the fixed cells awaiting staining. Optimal conditions were those which resulted in specific staining for NSE with minimal background staining. The steps involved in the optimisation of fixation and storage steps of the protocol are presented in Table 4.2.

Fixatives used	**Fixation times**	**Post-fixation storage**
ethanol 4% paraformaldehyde Bouins fixative neutral buffered formalin 0.2% glutaraldehyde/ 2% paraformaldehyde	all fixatives used for 10, 30 and 60 minutes	all combinations of fixatives and fixation times stored air-dried or in 70% ethanol

Table 4.2. Optimisation of cell fixation and storage for immunocytochemistry

NSE staining revealed that cells fixed in Bouins fixative for 10 minutes and subsequently stored in 70% ethanol gave the best ratio of neuronal to background staining of all the protocols tested. Cells processed in this way and stained for NSE are shown in figure 4.8a. This fixation time and method of post-fixation storage was kept constant throughout the remainder of the study since the neurons were well preserved in this protocol and clearly immunocytochemical staining of the neurons was possible. Figure 4.8c shows a representative culture stained for AVP with rabbit TG2-4 anti-AVP antibody. The staining pattern generated by the AVP antisera was non-specific as all the neurons in the culture were stained and the staining pattern was unaltered following preabsorption of the antibody with 5μM AVP (Figure 4.8d). The antibodies screened for identification of AVP neurons in hypothalamic culture and the immunocytochemical systems utilised are shown in Table 4.3. Whereas some antibodies tested resulted in non specific staining of all

Primary antibody	Host	Antibody type	Second antibody	Detection system	Substrate	Neurons stained
Arg^8-AVP	rabbit	polyclonal	SARB	AB-HRP	DAB	none
TG1 (6 bleeds screened)	rabbit	polyclonal	SARB	AB-HRP	DAB	all
TG2 (5 bleeds screened)	rabbit	polyclonal	SARB	AB-HRP	DAB	all
Mono 1	mouse	monoclonal	RAMB	AB-HRP	DAB	none
S277 (3 bleeds screened)	sheep	polyclonal	RASB	AB-HRP	DAB	all
S278 (3 bleeds screened)	sheep	polyclonal	RASB	AB-HRP	DAB	all
S279 (4 bleeds screened)	sheep	polyclonal	RASB	AB-HRP	DAB	all
4S41	sheep	polyclonal	RASB	AB-HRP	DAB	none
4S12	sheep	polyclonal	RASB	AB-HRP	DAB	none
PS41 anti- AVP specific neurophysin	mouse	monoclonal	RAMB	AB-AP	NBT	all
PS45 anti- AVP/ OT neurophysin	mouse	monoclonal	RAMB	AB-AP	NBT	all

Table 4.3. AVP antibodies screened for immunocytochemical identification of AVP neurons in culture.

SARB; swine anti-rabbit biotinylated
RAMB; rabbit anti-mouse biotinylated
RASB; rabbit anti-sheep biotinylated

the neurons in the culture, others did not appear to stain any of the cultured cells (data not shown).

Immunocytochemical staining for AVP-associated neurophysin using the alkaline phosphatase staining method is illustrated in Figure 4.8e. Omission of primary antibody was used as a negative control (4.8f). Staining with the antibody did not appear to be specific as all neurons in the culture were immuno-positive. Some neuronal cell bodies also appeared to be stained in the non-immune serum control.

Various methods were employed in an attempt to overcome the lack of success in identifying AVP neurons with immunocytochemistry. In order to capture the AVP neurons in a highly active state, cells were fixed mid-stimulation in an attempt to stimulate maximal amounts of AVP production. However, despite the fact that the cells were actively secreting AVP as demonstrated by radioimmunoassay, AVP neurons could not be identified immunocytochemically. Similarly, addition of a detergent step to the protocol in an attempt to increase the permeability of the neurons and therefore increase the access of the antibody to the antigen proved unsuccessful. In case the lack of AVP staining was a problem of visualisation, an immunofluorescent second antibody, swine anti-rabbit conjugated to fluoroscein isothiocyanate was used in the protocol in order to ease the visualisation of stained cells over background levels of staining. This too proved unsuccessful (data not shown).

4.4. Discussion

The objective of this study was to chart the growth and development of AVP neurons in cell culture in response to various neurotrophic factors. Functional maturation of the neurons was assessed in terms of the secretory response to potassium-induced depolarisation whilst development of neuronal morphology was to be monitored by immunocytochemistry. AVP secretion was consistently and repeatedly stimulated from cultured fetal rat hypothalamic neurons over the 20 day culture period. Treatment of the culture with 1, 10 or 100ng/ml IGF-1 in either insulin-containing or insulin-deficient medium had no significant effect on either basal or potassium-induced secretion of AVP at day 7, 10, 13, 16 or 20 of culture when compared to SFCM alone controls. Basal secretion of AVP remained at a constant level throughout the duration of the culture period. Despite the success of

the AVP antibodies in immunohistochemical identification of AVP in rat hypothalamic tissue sections, none of the antibodies screened in this study were able to specifically identify AVP neurons within the culture system.

Previous studies suggest that IGF-1 exerts a neurotrophic influence on hypothalamic neurons. Torres-Aleman *et al.* (1989) describe the growth promoting effects of IGF-1 on fetal rat hypothalamic cell lines. The authors described the potent influence of IGF-1 on cell growth as measured by cell counts and incorporation of ^{3}H-thymidine. Cultures treated with IGF-1 showed a dose-dependant growth response that was equal to or greater than that shown in response to insulin. The effect of IGF-1 on the cell lines was dependant upon continued presence of IGF-1 and withdrawal from the medium produced a decrease in the number of cells compared to continuos IGF-1 treated controls. Studies on fetal rat hypothalamic primary cultures (Torres-Aleman *et al.*, 1990) further substantiate the role of IGF-1 as a neurotrophic factor for hypothalamic neurons. IGF-1 was reported to significantly increase the survival of hypothalamic neurons and to promote the development of neurite-bearing cells within the culture. In addition, the increase in levels of the neuronal differentiation marker protein kinase C in IGF-1 treated cultures suggests that IGF-1 may be acting to accelerate the rate of neuronal differentiation. The results of the present functional study suggest that whilst IGF-1 may act as a neurotrophic factor for hypothalamic neurons, AVP neurons do not appear to be the specific target population for IGF-1 within the developing fetal hypothalamus. However, the inability to stain for AVP neurons within the culture system prevents the examination of any possible effects of IGF-1 on the rate of growth or the differentiated morphology of the AVP neurons.

The ability of the AVP antibodies used in the present study to immunohistochemically identify AVP neuronal fibres in the rat median eminence proves that the antisera were capable of staining for AVP, at least in the intact hypothalamus. However, despite extensive screening, none of the antibodies tested were able to identify AVP neurons within the culture system. One possibility for the lack of success in identifying AVP neurons in culture may be that the AVP neurons do not store sufficient amounts of peptide to be stained immunocytochemically. Whilst the neurons are actively secreting the peptide and may be stimulated to release AVP in response to potassium depolarisation, levels of AVP released into the medium are relatively small. Most cultures when maximally stimulated are

releasing only about 10pg/well and each well contains a total of $9x10^5$ cells, only a proportion of which are AVP neurons. Therefore, AVP neurons, even at times of stimulation do not appear to contain large amounts of AVP. Other workers have reported difficulties in immunocytochemical staining of AVP neurons in cell culture (Denizeau, Dube, Antakly, Lemay, Parent, Pelletier and Labrie, 1981). In accordance with the present study, Denizeau *et al.* (1981) were able to measure AVP release into culture medium but were unable to identify AVP neurons in 3 to 7-day old cultures using immunocytochemical techniques. Positive identification of AVP neurons in dissociated cell culture has been described (Jirikowski, Reisert and Pilgrim, 1981). However, in this study the authors were able to identify AVP neurons in only 4 out of 8 cultures examined and then only 1.1% of the $3x10^6$ hypothalamic cells plated onto each dish were immunopositive. This contrasts considerably with the present study in which hypothalamic neurons were non-specifically stained. However, it may go some way towards explaining the lack of staining in response to some of the antibodies tested. The unsuccessful attempts at immunocytochemical detection suggest that the neurons maintained in hypothalamic culture do not store significant amounts of AVP and that the amounts which are present are below the levels of sensitivity of the immunocytochemical technique.

AVP peptide is produced in the cell body in association with a specific carrier protein, AVP-specific neurophysin. The peptide and neurophysin are encoded on the same gene and translated together to form a precursor molecule (Gainer, Altstein, Whitnall and Wray, 1988). The prohormone molecule is transported to the nerve terminals where endoproteolytic cleavage enzymes act to excise the peptide from the precursor. It is possible that the inability to detect AVP within the primary culture system reflects the inability of the antibody to access AVP in the AVP-neurophysin precursor molecule. The anti-neurophysin antibodies used in this study had previously been characterised in tissue sections of fetal rat hypothalami (Ben-Barak, Russell, Whitnall, Ozato and Gainer, 1985; Whitnall *et al.*, 1985) and recognises both the AVP-NP molecule and the prohormone precursor. Immunoreactivity for AVP-NP was first identified at embryonic day 16 and levels increased throughout fetal life. The antibody stained neuronal fibres in both the internal and external zone of the median eminence illustrating the presence of AVP-NP, or the precursor molecule in the neural processes of the hypothalamic cells and the ability of the antibody to stain AVP neurons of both magnocellular and parvocellular origin. In studies of slice explant culture, Gainer and Wray (1992)

reported that only 20% of cultures encompassing the paraventricular nucleus contained identifiable AVP neurons and of those that did only 1-3 cells per culture were immunopositive. In the present study, attempts to immunocytochemically stain for the AVP-specific neurophysin molecule within the cell cultures proved unsuccessful. The reason for the lack of success with the neurophysin antibody is unclear. In contrast to the very low levels of positive staining described by Gainer and Wray (1992), in the present study all neurons in the culture were immunopositive. This perhaps suggests that the integrity of the neuronal cells is somehow undermined by the dispersion procedure, resulting in unlimited access of the antibodies and non-specific staining. It is of interest that in the stained cultures, only the neurons display this high level of non-specific staining with the underlying glial cells remaining negative. The identification of underlying cells within the culture system as glial cells was determined by immunocytochemical detection of the glial specific factor, glial fibrillary acidic protein (GFAP; data not shown). This suggests that whatever mechanisms are involved in conferring positive immunoreactivity to all the neuronal cells within the dispersed culture system is neuron-specific.

In conclusion, the study presented in this chapter demonstrates that AVP neurons of the fetal hypothalamus do not represent a specific target population for IGF-1 in terms of functional maturation. However, the inability to identify specific AVP containing neurons within the culture system makes it impossible to determine whether IGF-1 influences the growth and morphological development of these neurons.

Chapter 5. Ontogeny of POMC gene expression and expression of translated products ACTH and α-MSH in the ovine fetal pituitary gland.

5.1. Introduction

The polypeptide precursor gene proopiomelanocortin (POMC) is expressed in both the corticotrophs of the pars distalis and the melanotrophs of the pars intermedia and is differentially processed such that ACTH, β-endorphin and β-lipotrophin are the major POMC-derived products of the anterior pituitary while melanotrophs synthesise mainly α-MSH, CLIP and acetylated β-endorphin. In the ovine fetus, POMC mRNA has been detected as early as day 60 gestation by Northern analysis (McMillen *et al.*, 1988; Yang *et al.*, 1991). Recently, attempts have been made to determine whether or not the late gestation increase in ACTH in fetal circulation in accompanied by an increase in pituitary POMC gene expression. However, the technique of Northern analysis has produced conflicting data concerning the ontogeny of POMC gene expression in the fetal sheep pituitary with some workers reporting an increase in POMC expression with advancing gestation (Yang *et al.*, 1991; Myers *et al.,* 1992) whilst others report a decline in levels as term approaches (McMillen *et al.*, 1988; Brooks *et al.*, 1992). The more sensitive technique of in situ hybridisation allows a more detailed analysis of the cellular localisation of messenger RNA encoding the POMC gene to be studied. Using this technique, Myers *et al.* (1993) reported a significant increase in POMC expression levels in the ovine fetus between days 105-107 and days 138-140 gestation. During the course of this thesis, Matthews *et al.* (1994) used in situ hybridisation to study the ontogeny and distribution of POMC in the ovine fetus and reported, in accordance with studies of Northern analysis that POMC mRNA is detectable from day 60 gestation with levels increasing to term. These authors also reported a regional distribution of POMC mRNA in the anerior lobe of the pituitary gland, with significantly greater levels of expression in the region around the base of the gland when compared to the region adjacent to the intermediate lobe and described differential expression of POMC mRNA in the two regions as gestation proceeds.

In addition, four POMC-derived peptides, ACTH, β-endorphin/β-lipotrophin, proγMSH and γMSH can been detected in fetal anterior pituitary cells by

immunohistochemistry at day 38 gestation (Mulvogue *et al.*, 1986) inferring that the POMC gene is actively transcribed and translated very early in gestation.

In this chapter, the ontogeny of POMC gene expression from a much earlier gestational age (day 30) to term was studied by in situ hybridisation. In addition, the ontogeny of expression of the POMC-derived peptides α-MSH and ACTH was determined by immunohistochemistry.

5.2. Materials and Methods

5.2.1. Animals and tissue collection

29 sheep with known insemination dates were used in this study. At the appropriate gestational age, ewes were killed with an overdose of anaesthetic and fetuses delivered as described previously (see Chapter 3). Where the ewe was pregnant with twins, both fetal pituitaries were collected. Fetal pituitaries were collected at day 30 (n=6), day 40 (n=6), day 50 (n=3), day 70 (n= 3), day 100 (n=2), day 134 (n=4) and day 141 (n=3) gestation. At day 30 gestation the intact fetus was fixed in 4% paraformaldehyde. After fixation, the fetus was decapitated and the whole fetal head was processed to paraffin wax. At day 40 and day 50 gestation, intact fetuses were collected, decapitated and the fetal head was fixed in its entirety. At day 50 gestation, the top of the skull was removed to expose the fetal brain prior to fixation. Day 40 gestation fetal heads were processed to paraffin wax and sectioned intact whilst at day 50 gestation pituitaries were isolated under a dissecting microscope after fixation and the isolated pituitaries were processed. All isolated pituitaries from day 70 gestation to adults were halved in the coronal plane before processing to paraffin wax and sectioning as described in section 3.6. In order to allow for comparison between gene expression and localisation of translated POMC-derived peptides, consecutive sections were processed for in situ hybridisation and immunohistochemistry.

5.2.2. Northern analysis

RNA was extracted from fetal sheep pituitaries at day 70, day 100 and day 130 gestation, subjected to electrophoresis on 1.5% agarose gel and transferred to nylon membrane by capillary blotting as described in section 3.8. The Northern blot was then hybridised for 24 hours at 65° with [^{32}P] POMC riboprobe, washed and

exposed to X-ray film according to the method described in section 3.11. As an internal control for efficiency of RNA transfer, blots were stripped with several changes of boiling 0.1 x SSC/ 0.5% SDS for 30 minutes and hybridised with end labelled labelled [^{32}P] 18S rRNA oligonucleotide (section 3.10).

5.2.3. Radioactive in situ hybridisation for POMC

Radiolabelled [^{35}S] POMC riboprobe was prepared as described in section 3.12 and radioactive in situ hybridisation for POMC was carried out as described in section 3.13. For each pituitary in the study 8 tissue sections were mounted as 2 sections per slide on 4 separate slides and were treated with antisense riboprobe. 2 slides containing 2 tissue sections from each pituitary were treated with sense riboprobe as a negative control. Preliminary experiments revealed that POMC expression in pars distalis and pars intermedia was optimal at two different exposure times and so half of the antisense and sense hybridised slides were developed after 3 days for determination of pars intermedia gene expression and the other half developed after 10 days for determination of pars distalis POMC gene expression.

5.2.4. Immunohistochemistry

Immunohistochemical detection of α-MSH and $ACTH_{(18-39)}$ was performed using the avidin-biotin staining procedure described in section 3.7.

5.2.5. Data analysis

All tissues in the study were processed together to allow for direct comparison between gestaional ages. Each slide in the study contained a tissue section from an adult sheep pituitary as a positive control and to ensure standardisation of the procedure between slides. Sections were analysed using a computerised image analysis program (TCL-image, Multihouse, Amsterdam) for the Macintosh II computer which detects the number of autoradiographic silver grains in any given area. Data are presented as the number of silver grains per 100μm^2. The position in the pituitary at which the analysis was undertaken was consistent throughout. As POMC gene expression in the pars distalis is regionally distributed, the area around the intermediate lobe and the area at the base of the anterior lobe were analysed separately. A minimum of 3 separate tissue sections were analysed from each pituitary in the study and 5 fields were counted for each area in the tissue to be

analysed. For cell counting of corticotrophs, a minimum of two tissue sections from 2-5 different pituitaries from each gestational age were counted. Four fields from the corticotroph dense region at the base of the anterior lobe were counted on each section. For all photographs; AL, anterior lobe, NL, neural lobe and IL, intermediate lobe.

5.3. Results

5.3.1. Identification of POMC mRNA by Northern analysis

Figure 5.1 shows a Northern blot of pituitary RNA extracts hybridised with radiolabelled POMC riboprobe. A single RNA transcript was identified at all ages examined. Hybridisation with 18S rRNA demonstrates that RNA was evenly loaded and efficiently transferred to the Northern blot.

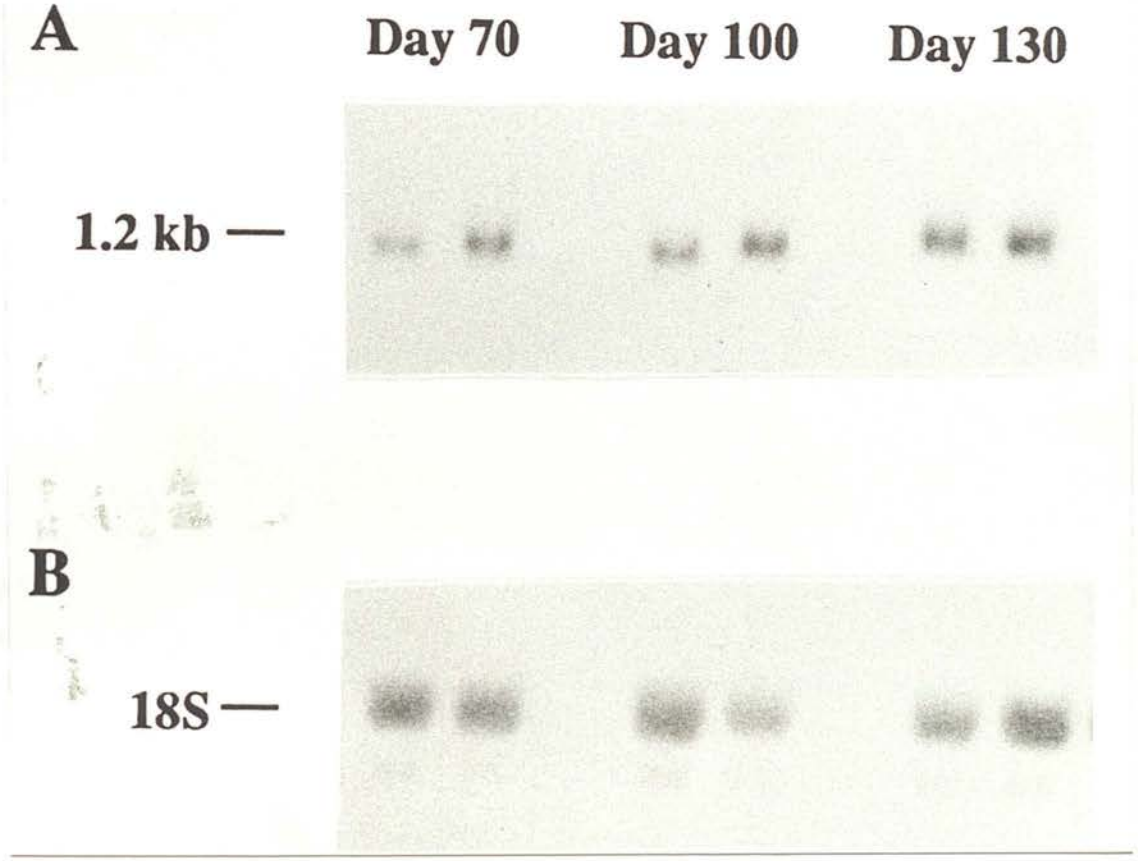


Figure 5.1. A. Northern blot analysis of total RNA extracts from fetal pituitaries at day 70, 100 and 130 gestation hybridised with [^{32}P] POMC riboprobe. 15µg total pituitary RNA was loaded onto each lane. A single transcript of approximately 1.2kb was identified at all three gestational ages. B. The same blot was hybridised with 18S rRNA as a control for even lane loading and efficiency of transfer.

5.3.2. Cellular localisation of POMC, ACTH and α-MSH

Figure 5.2 shows a representative section of day 70 fetal pituitary hybridised with a) POMC antisense and b) POMC sense riboprobe. Antisense probe showed specific hybridisation to the intermediate lobe but not to the neural lobe. There was no specific hybridisation to pituitary tissue with the sense riboprobe.

Day 30 gestation

At day 30 gestation, POMC mRNA was undetectable by in situ hybridisation in the developing pituitary gland (Figure 5.3). Similarly, α–MSH and ACTH immunoreactivity were not detectable at day 30 gestation (Figure 5.4).

Day 40 gestation

At day 40 gestation, POMC mRNA was present in both the anterior and intermediate lobes of the pituitary (Figure 5.5). In the anterior pituitary, POMC expressing cells were arranged in cords whereas those in the intermediate pituitary tended to be less uniformly distributed. Both α-MSH and ACTH were detectable by immunohistochemistry in the anterior pituitary (Figure 5.6). Immunopositive cells were arranged in cords, consistent with the pattern of POMC gene expression. A few α-MSH and ACTH immunopositive cells were detected in the intermediate pituitary. These cells were always found in the innermost layer of the intermediate lobe, that is the layer of cells adjacent to the neural lobe.

Day 50 gestation

POMC mRNA was detectable in both the anterior and intermediate lobes of the pituitary at day 50 gestation (Figure 5.7). POMC mRNA in the intermediate lobe appeared as a narrow band of expression whilst the pattern of expression in the anterior pituitary was more diffuse. ACTH immunopositive cells were detectable in both the anterior and intermediate lobes (Figure 5.8). Corticotrophs in the anterior pituitary were arranged in cords or palisades and immunopositive cords of cells were found throughout the gland separated by areas of vascular mesenchyme. α-MSH immunoreactivity was confined to the intermediate lobe in 2 of the day 50 pituitaries analysed however, in one pituitary some α-MSH immunopositive cells were identified in the anterior lobe (Figure 5.8A, arrows). As at day 40 gestation, intermediate lobe cells immunopositive for α–MSH and ACTH tended to border

Figure 5.2. Demonstration of probe specificity in consecutive coronal sections of intermediate pituitary at day 70 gestation. Tissue sections were hybridised with ^{35}S labelled- A) POMC antisene and B) POMC sense riboprobe. Specific hybridisation was only found with the antisense riboprobe. A and B x10 magnification.

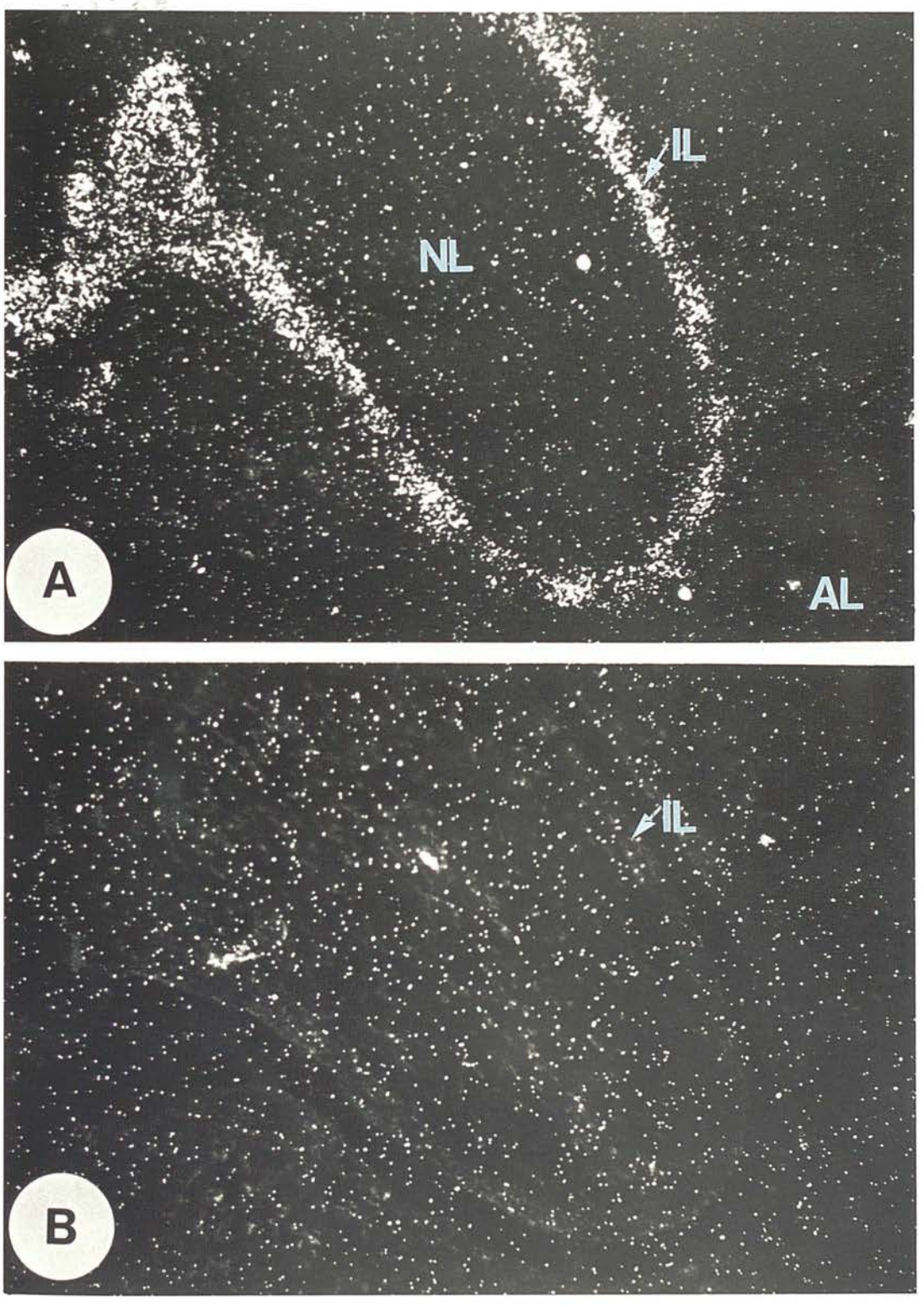

IL
NL
A
AL
IL
B

Figure 5.3. In situ hybridisation for POMC mRNA in a representative pituitary at day 30 gestation shown in A) darkfield and B) brightfield photomicrographs. No specific hybridisation was detected at this gestational age. A and B x10 magnification.

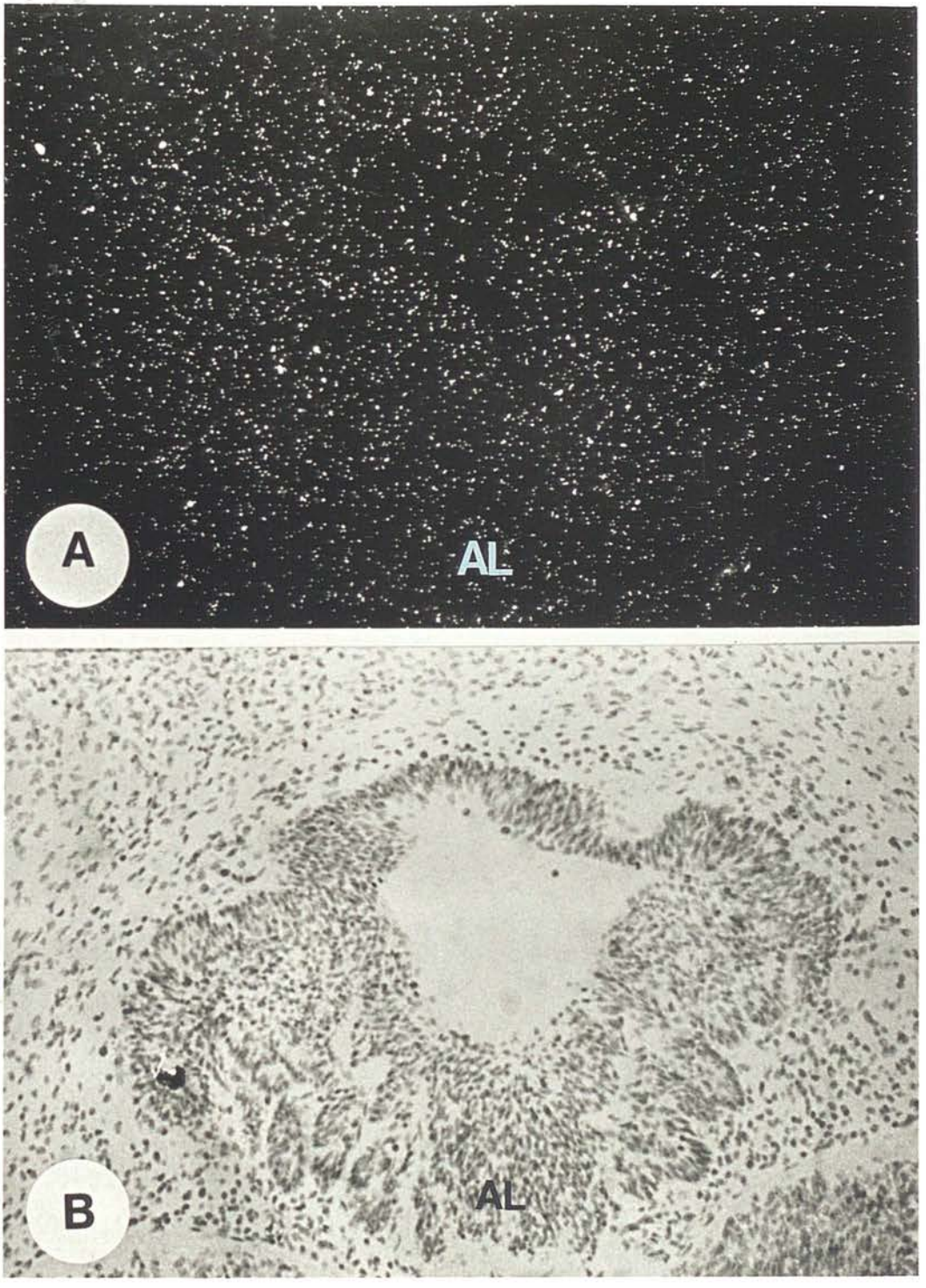
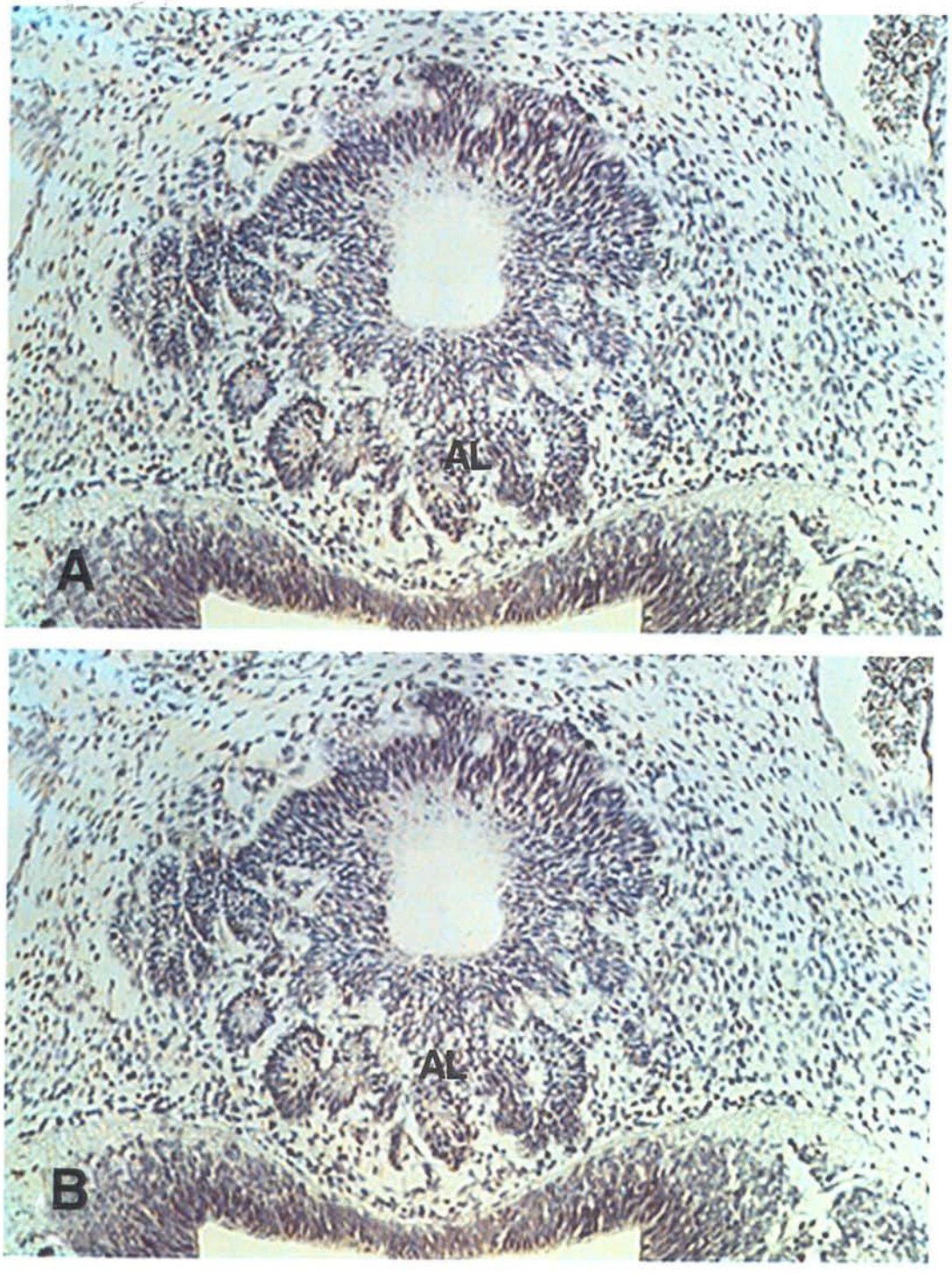

A
AL
B
AL

Figure 5.4. Immunohistochemical staining for A) α-MSH and B) ACTH in a representative pituitary at day 30 gestation. No immunopositive cells were detected at this gestational age. A and B x10 magnification.


AL
A
AL
B

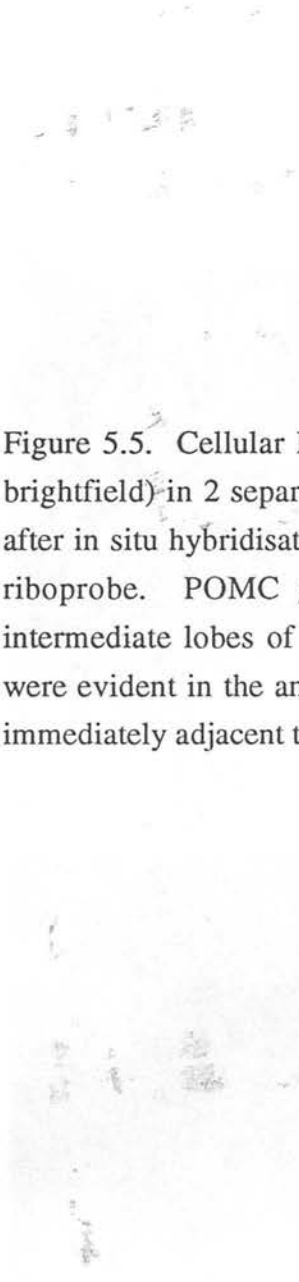


Figure 5.5. Cellular localisation of POMC mRNA (A and C darkfield; B and D brightfield) in 2 separate fields from a representative pituitary at day 40 gestation after in situ hybridisation of coronal sections with a ^{35}S-labelled POMC antisense riboprobe. POMC gene expression was detected in both the anterior and intermediate lobes of the pituitary. Palisades of cells expressing POMC mRNA were evident in the anterior pituitary (5.5C). Expression was absent in the region immediately adjacent to the intermediate lobe (arrows). All x10 magnification.

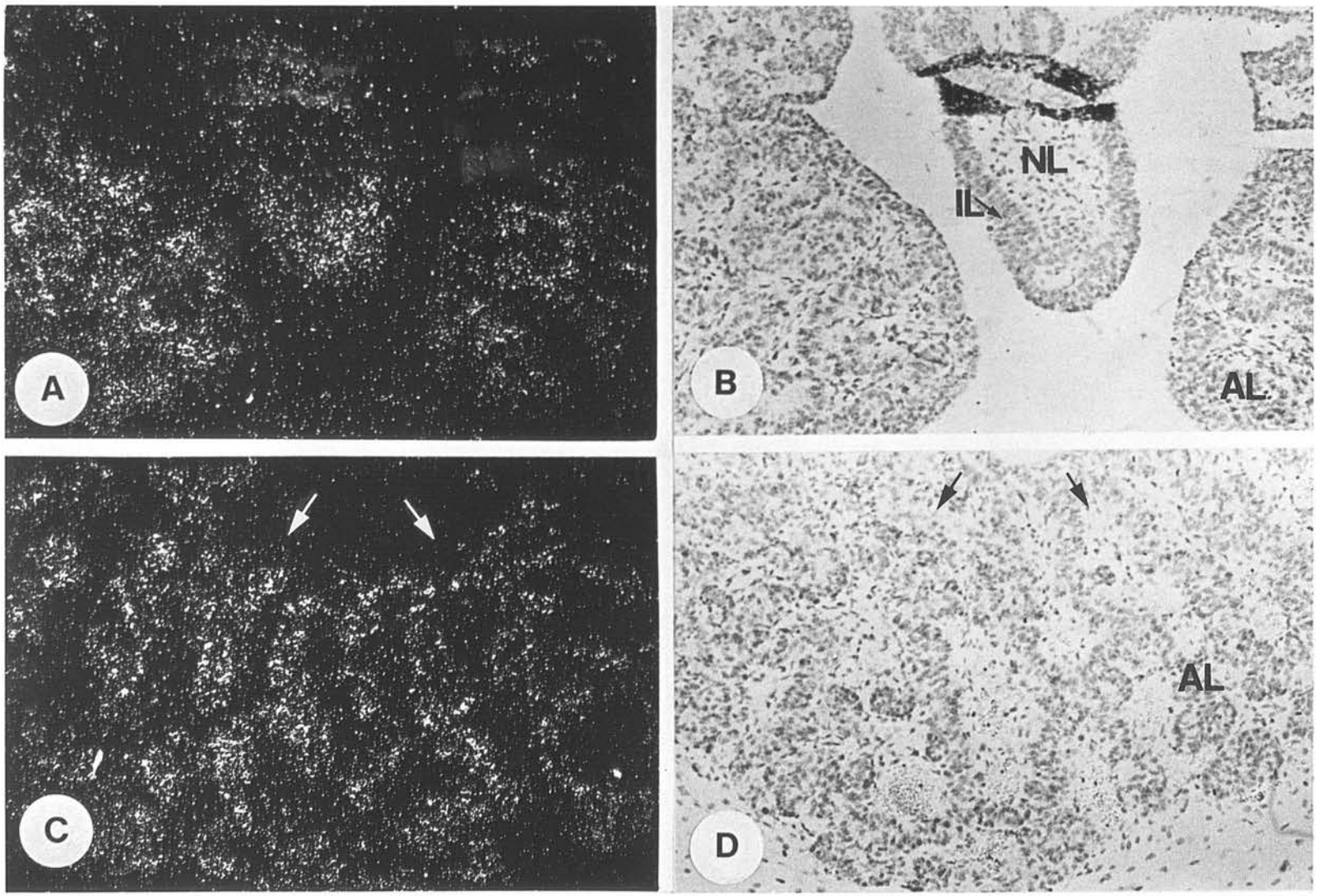
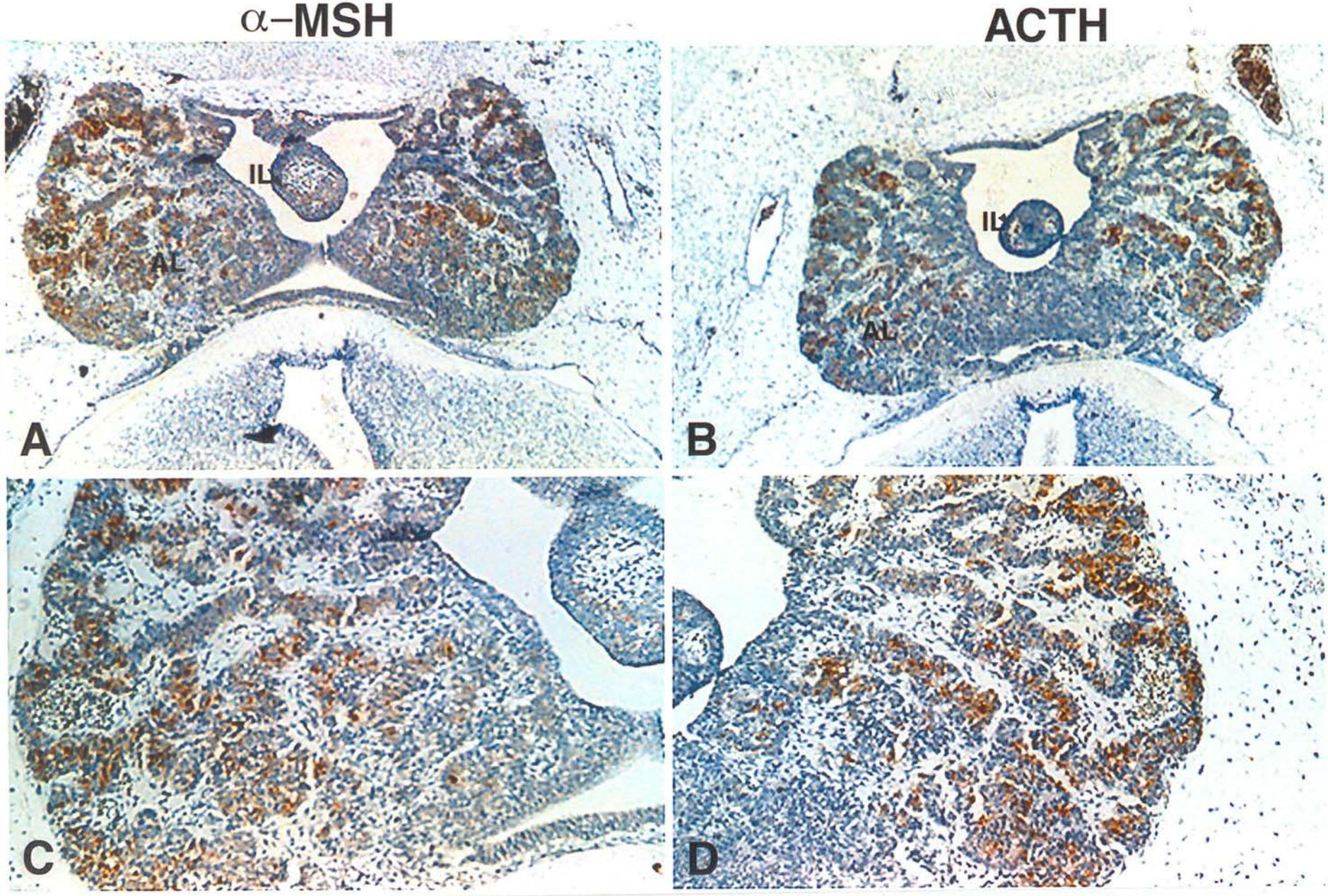

A
B
NL
IL
AL
C
D
AL

Figure 5.6. Immunohistochemical detection of α-MSH (A and C) and ACTH (B and D) in coronal sections of a representative pituitary at day 40 gestation. α-MSH and ACTH were detected in both the anterior and intermediate lobes of the pituitary. Immunopositive cells in the intermediate lobe were confined to the layer of cells immediately adjacent to the neural lobe. Both α-MSH and ACTH were abundantly expressed in the anterior pituitary. A and B x6.5, C and D x15 magnification.


α–MSH
ACTH
IL
AL
IL
AL
A
B
C
D

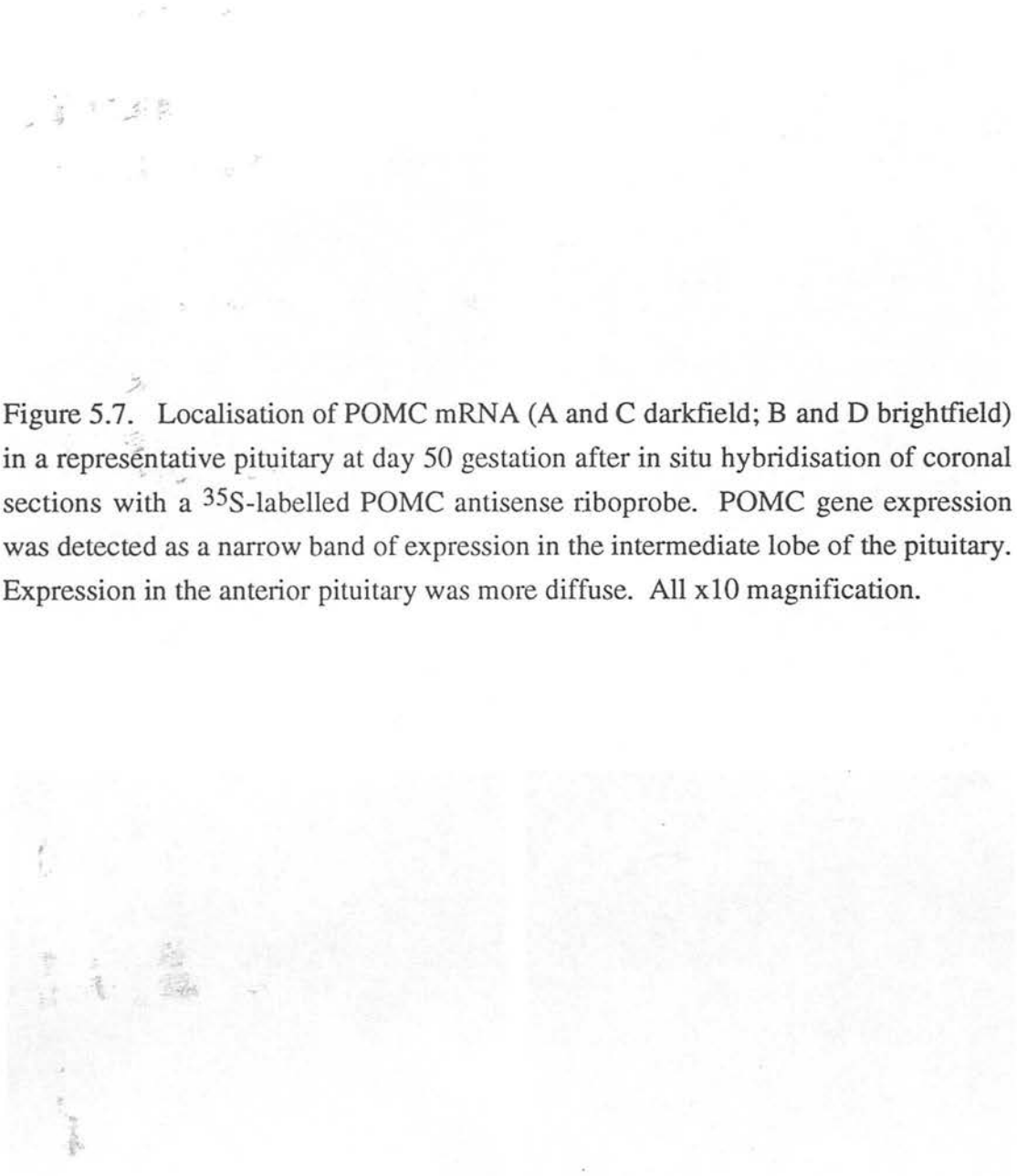
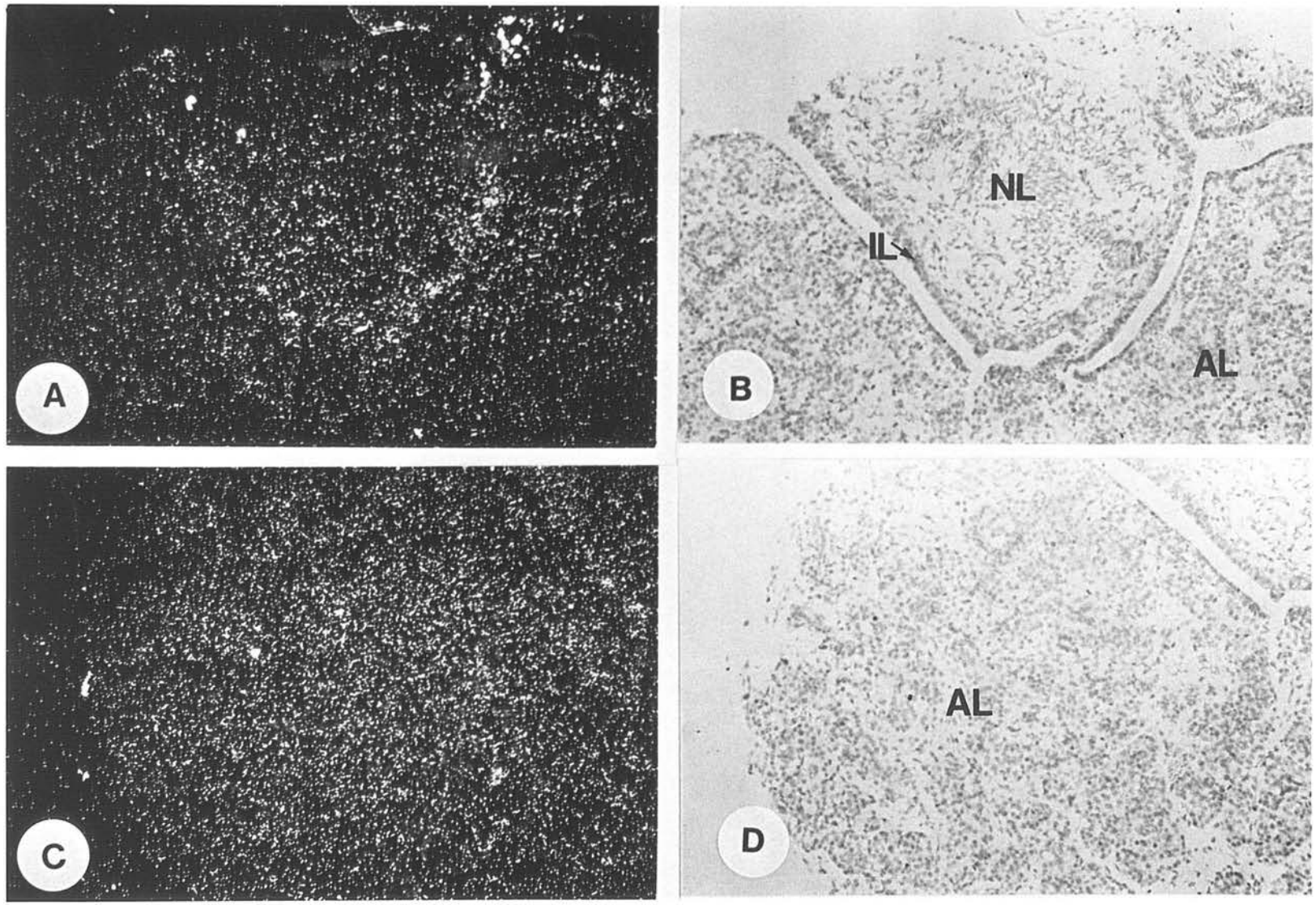


Figure 5.7. Localisation of POMC mRNA (A and C darkfield; B and D brightfield) in a representative pituitary at day 50 gestation after in situ hybridisation of coronal sections with a ^{35}S-labelled POMC antisense riboprobe. POMC gene expression was detected as a narrow band of expression in the intermediate lobe of the pituitary. Expression in the anterior pituitary was more diffuse. All x10 magnification.


A
B
NL
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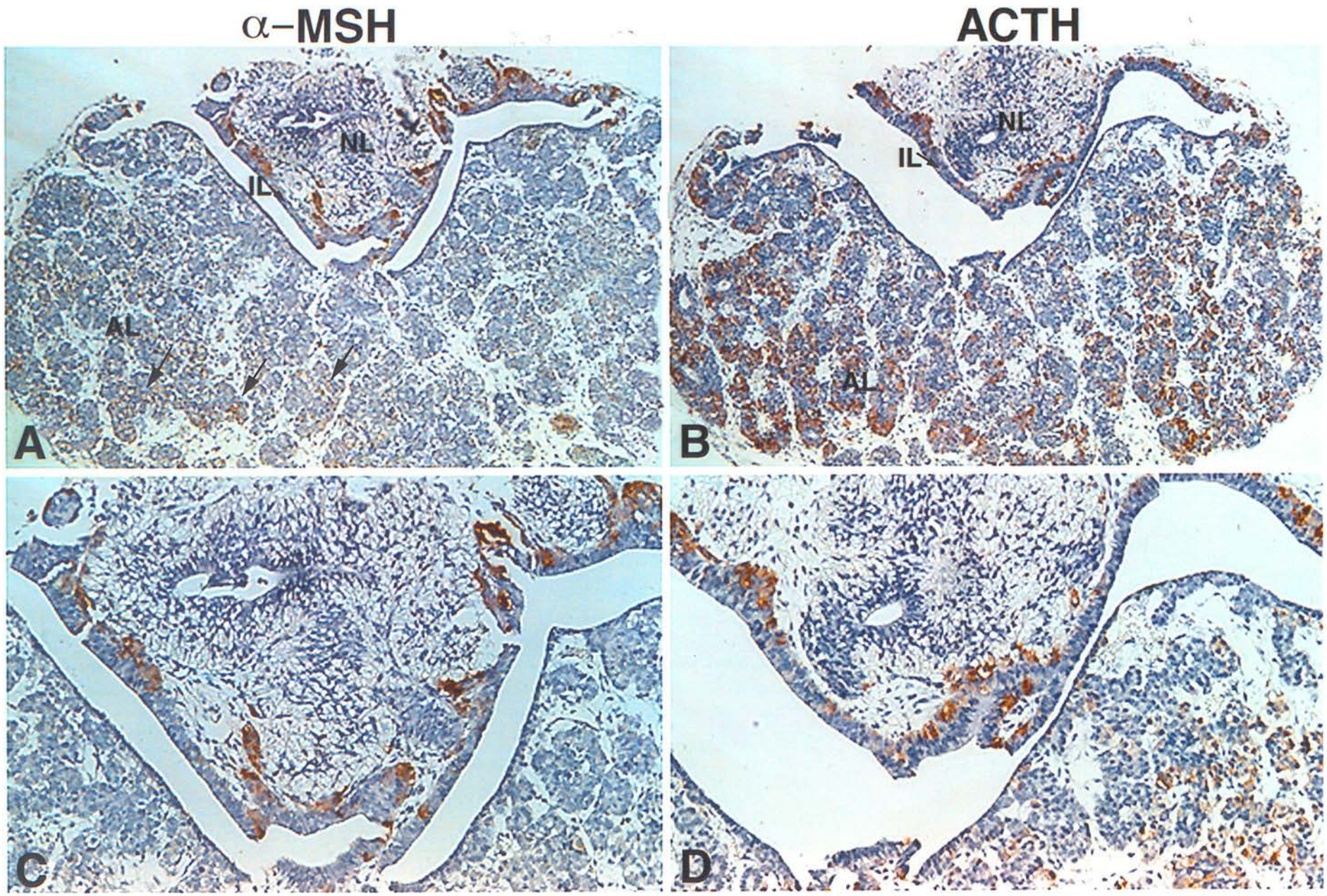
AL
C
D
AL

Figure 5.8. Immunohistochemical detection of α-MSH (A and C) and ACTH (B and D) in coronal pituitary sections at day 50 gestation. α-MSH was largely localised to the layer of intermediate lobe cells adjacent to the neural lobe. In the pituitary shown, a few faintly stained α-MSH immunopositive cells were found in the anterior lobe (5.8A, arrows). ACTH was clearly detectable in both the anterior and intermediate lobes of the pituitary. Immunopositive cells in the intermediate pituitary are confined to the cell layer adjacent to the neural lobe. A and B x10, C and D x 20 magnification.


α–MSH
ACTH
NL
IL
AL
A
NL
IL
AL
B
C
D

with the neural lobe with those cell layers closest to the hypophysial cleft remaining unstained.

Day 70 gestation

By day 70 gestation, POMC mRNA was abundantly expressed in the intermediate lobe of the pituitary (Figure 5.9). In the anterior pituitary, some cord-like expression of the gene was evident however, the cord were shorter than at earlier gestational ages and gave the appearance of small clusters of expression. Immunohistochemical localisation of α-MSH revealed specific staining confined to the intermediate lobe of the pituitary (Figure 5.10). Occasional immunostaining for α-MSH evident in the anterior lobe was confined to blood vessels. As at earlier gestational ages, the immunopositve cells were localised around the neural lobe with those cells closest to the anterior lobe of the pituitary remaining unstained. ACTH immunoreactive cells were identified in both the intermediate and anterior lobes of the pituitary. In the intemediate lobe, ACTH immunoreactive cells were distributed like those immunopositive for α-MSH, with those cells closest to the anterior pituitary remaining unstained. In the anterior pituitary, ACTH immunopositive cells were found in cords or small clusters in keeping with the pattern of POMC gene expression, with the region immediately adjacent to the intermediate lobe largely devoid of expression.

Day 100 gestation

POMC mRNA was abundantly expressed throughout the intermediate pituitary at day 100 gestation, and almost all cells within the lobe appeared to express the POMC gene (Figure 5.11). In the anterior pituitary, POMC mRNA was localised in clusters of varying size which tended to occupy the basal aspect of the gland. Immunohistochemical staining for α-MSH and ACTH revealed that staining in the intermediate lobe differed significantly at day 100 when compared to day 70 gestation, with immunopositive cells occupying all of the layers of the intermediate lobe, including those layers closest to the anterior pituitary (Figure 5.12). There was no detectable α-MSH staining in the anterior lobe. ACTH immunoreactive cells in the anterior pituitary were evident as single cells or as weakly stained clusters or palisades.

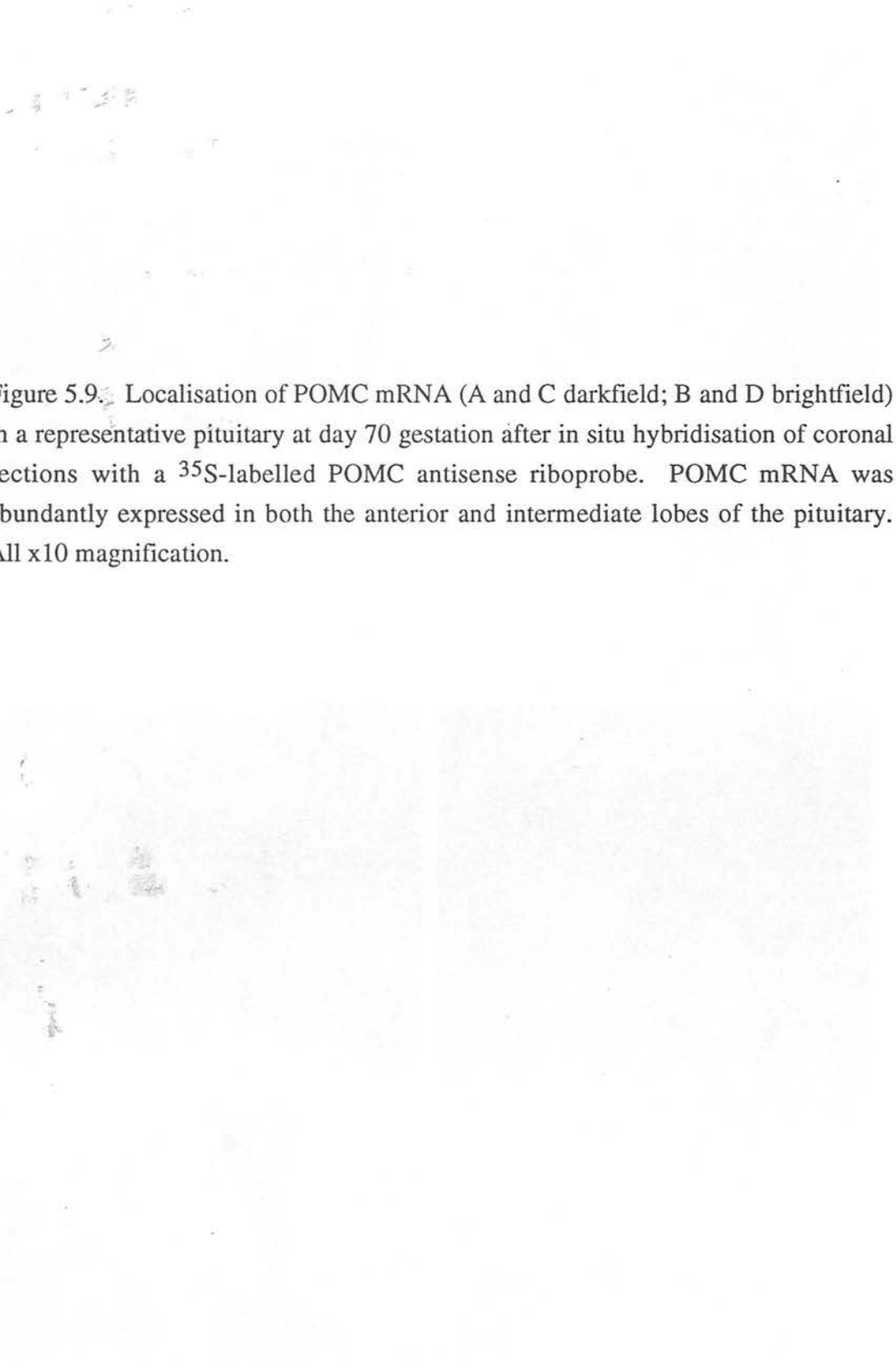


Figure 5.9. Localisation of POMC mRNA (A and C darkfield; B and D brightfield) in a representative pituitary at day 70 gestation after in situ hybridisation of coronal sections with a ^{35}S-labelled POMC antisense riboprobe. POMC mRNA was abundantly expressed in both the anterior and intermediate lobes of the pituitary. All x10 magnification.

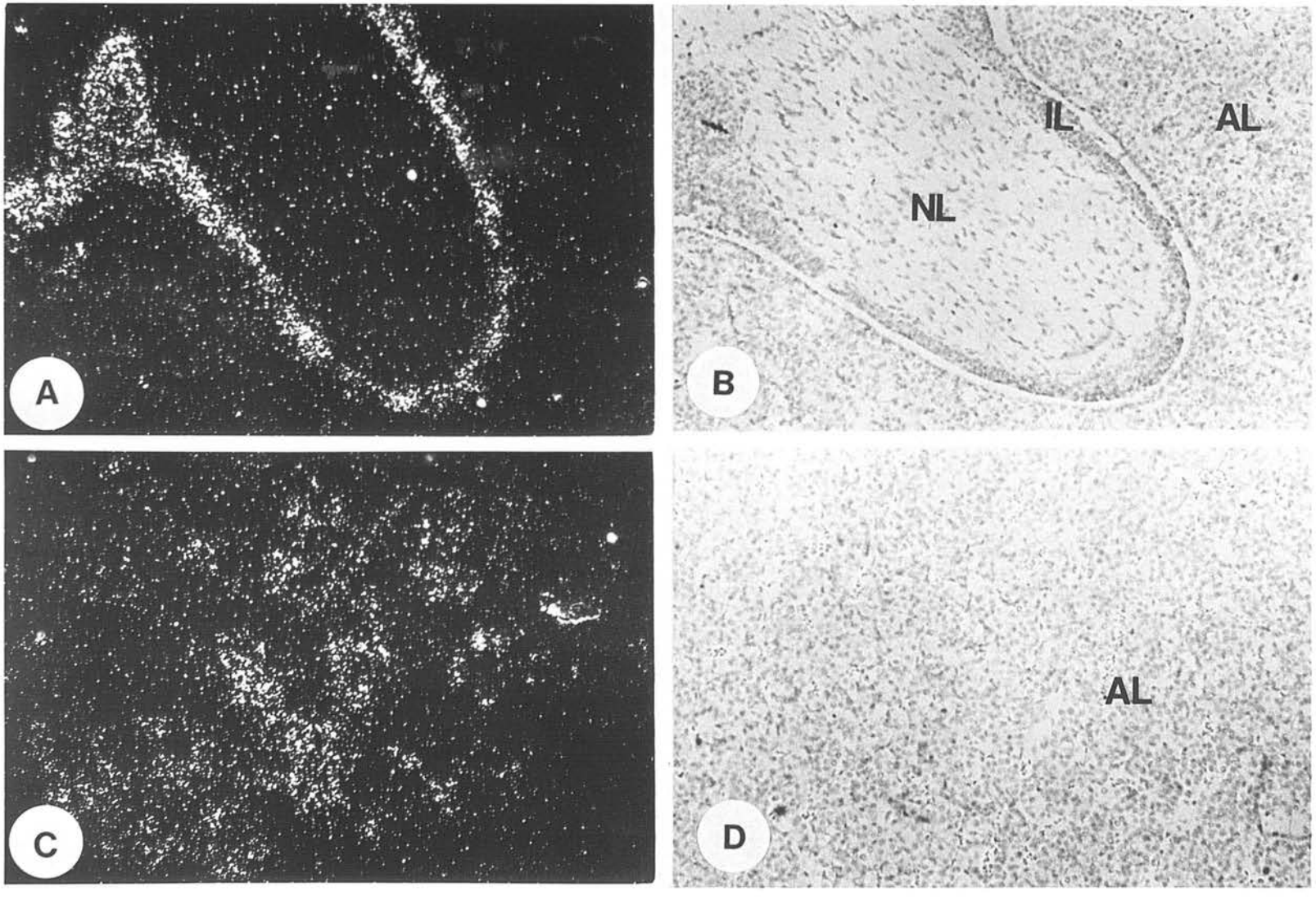
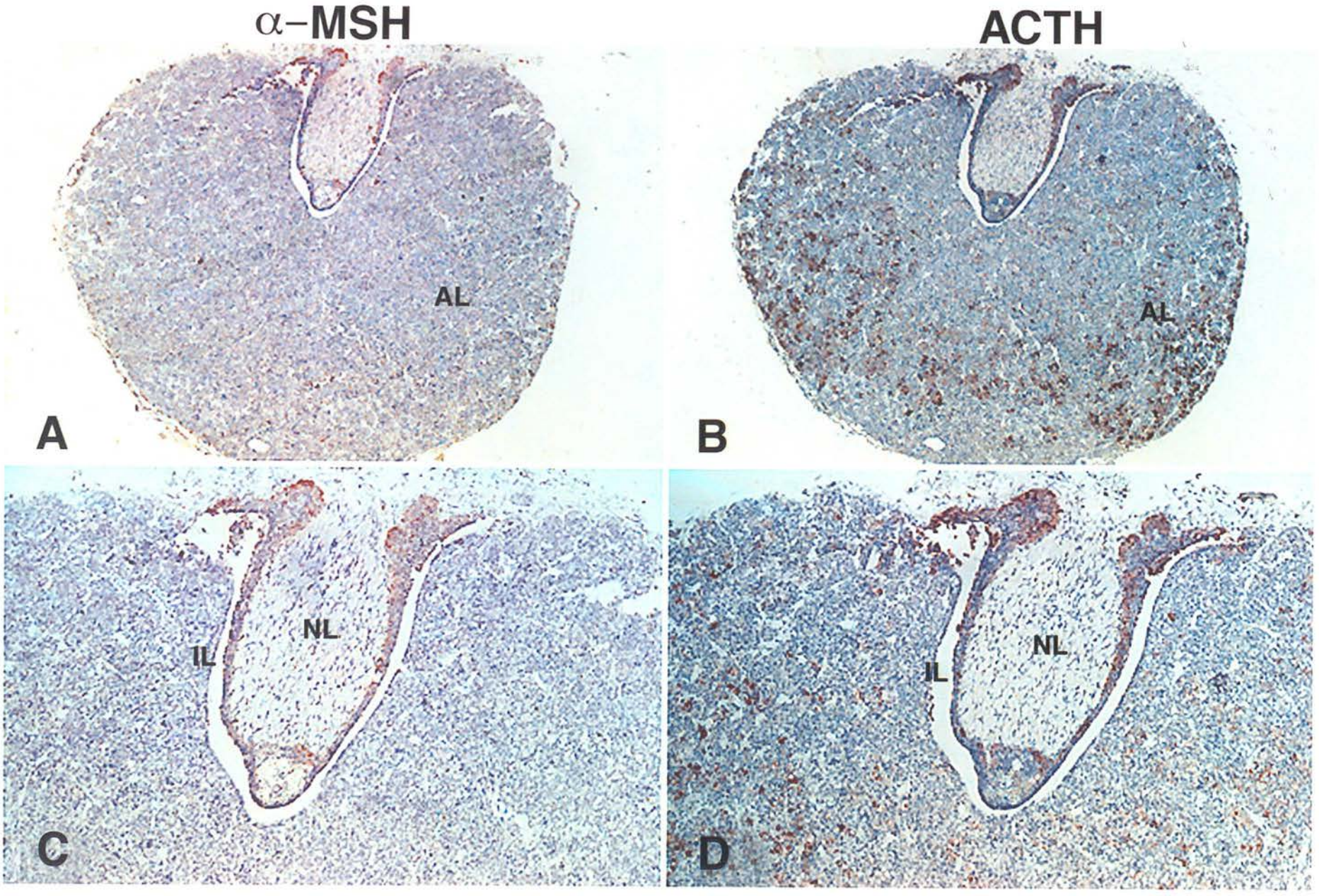

A
B
IL
AL
NL
C
D
AL

Figure 5.10. Immunohistochemical detection of α-MSH (A and C) and ACTH (B and D) in coronal sections of a representative pituitary at day 70 gestation. α-MSH immunostaining was confined to the intermediate lobe. The occassional presence of α-MSH immunostaining in the anterior lobe was confined to blood vessels. ACTH was detected in both the anterior and intermediate lobes of the pituitary. Immunostaining in the intermediate lobe was localised to the innermost cell layers, with those layers closest to the hypophysial cleft remaining unstained. A and B x4, C and D x10 magnification.


α–MSH
ACTH
AL
AL
A
B
IL
NL
IL
NL
C
D

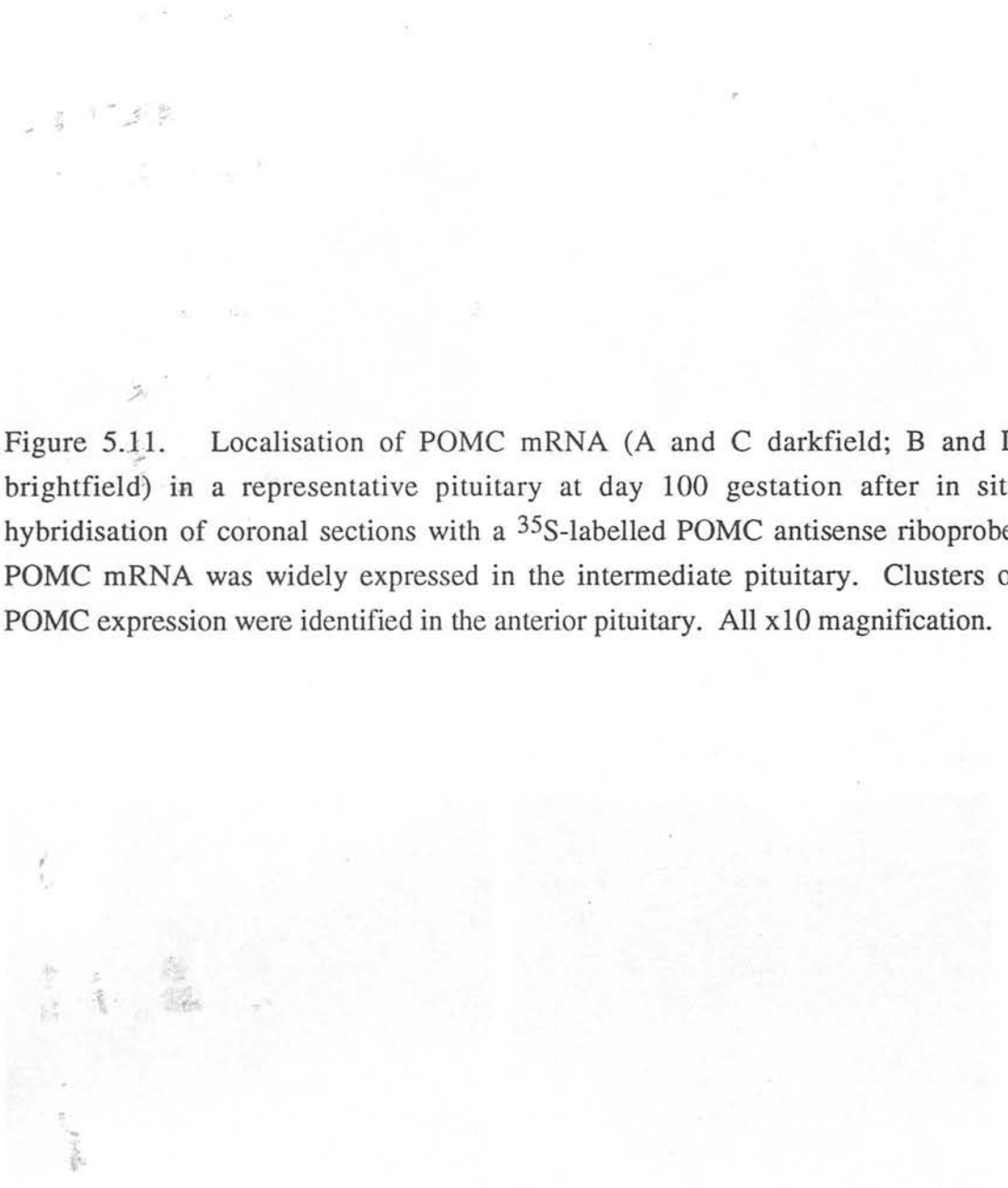


Figure 5.11. Localisation of POMC mRNA (A and C darkfield; B and D brightfield) in a representative pituitary at day 100 gestation after in situ hybridisation of coronal sections with a ^{35}S-labelled POMC antisense riboprobe. POMC mRNA was widely expressed in the intermediate pituitary. Clusters of POMC expression were identified in the anterior pituitary. All x10 magnification.

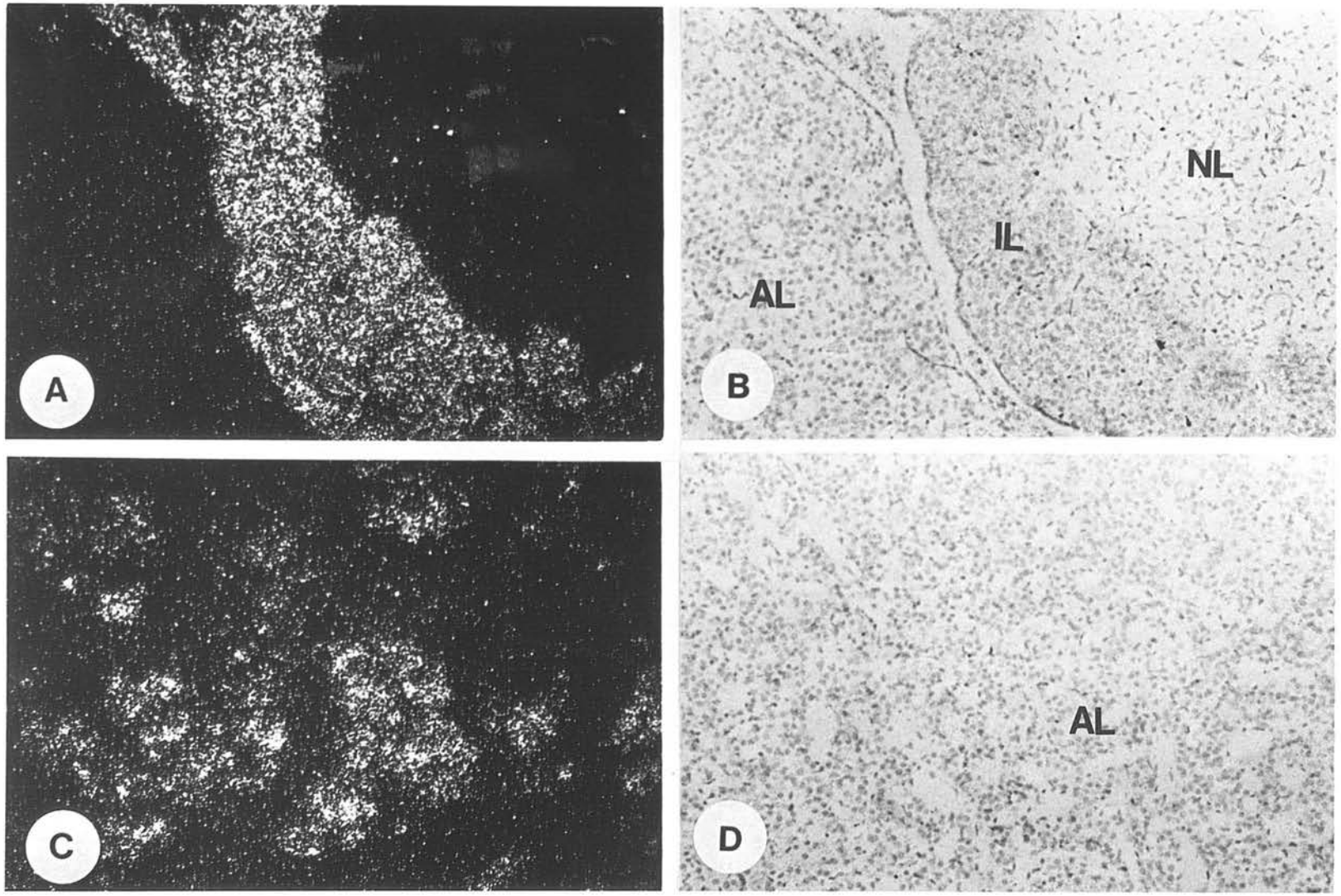
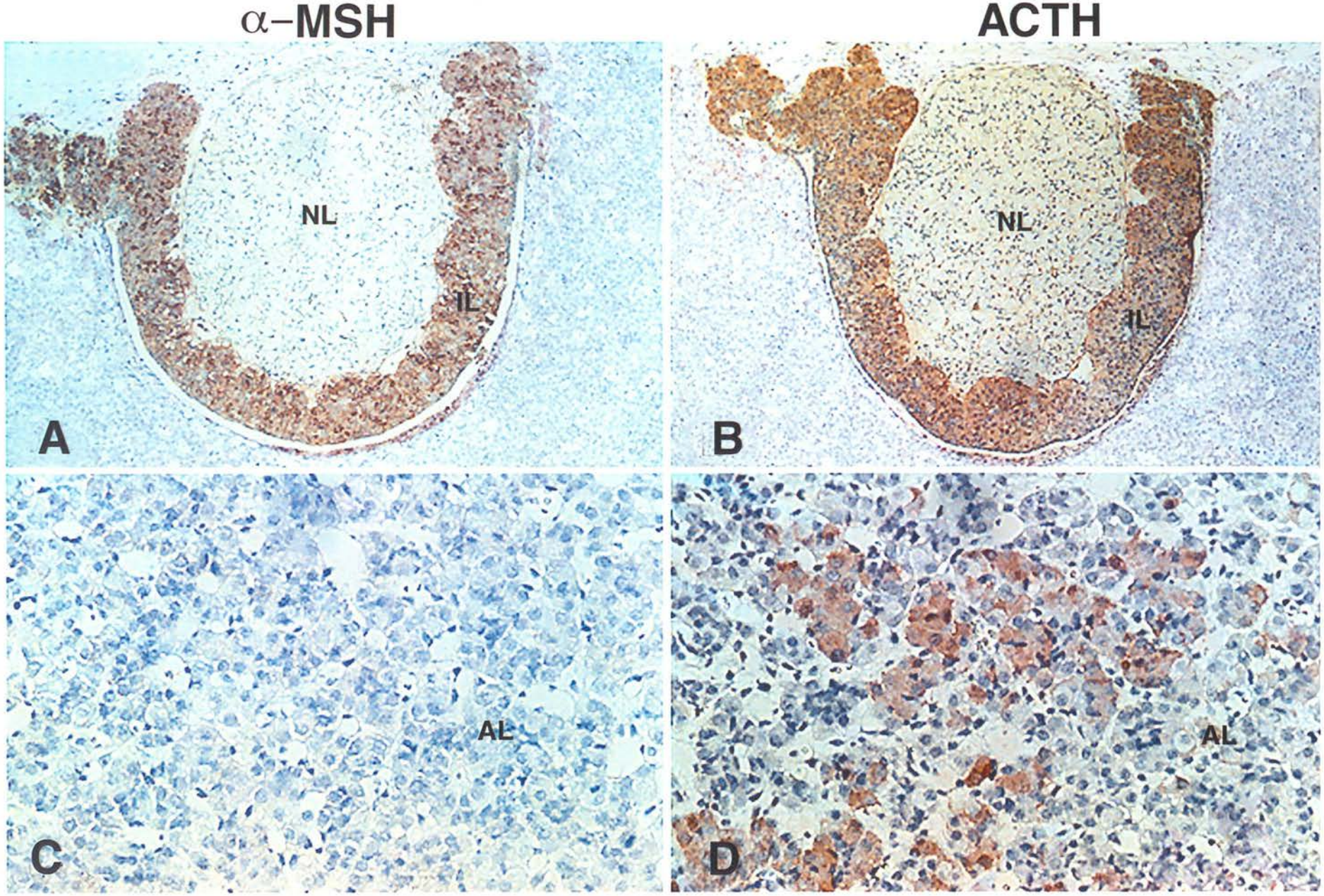

A
B
NL
IL
AL
C
D
AL

Figure 5.12. Immunohistochemical detection of α-MSH (A and C) and ACTH (B and D) in coronal sections of a representative pituitary at day 100 gestation. α-MSH immunostaining was confined to the intermediate lobe. ACTH was detected in both the anterior and intermediate lobes of the pituitary. Immunostaining for α-MSH and ACTH was distributed throughout the cell layers of the intermediate lobe. A and B x 10, C and D x30 magnification.


α–MSH
ACTH
NL
IL
NL
IL
A
B
AL
AL
C
D

Day 134 gestation

The cellular localisation of POMC mRNA at day 134 gestation was not dissimilar to day 100 gestation, with the intermediate lobe of the pituitary abundantly expressing the POMC gene and many small clusters of expression evident in the anterior pituitary (Figure 5.13). α-MSH and ACTH were detected by immunohistochemistry throughout the intermediate lobe and ACTH immunopositive cells were present as clusters in the anterior pituitary (Figure 5.14). ACTH immunopositive cells in the anterior pituitary appeared morphologically different from those seen at earlier gestational ages. Individual corticotrophs had a more distinct shape compared to the palisade-like appearance of corticotrophs at earlier gestational ages, however, some palisades of ACTH immunopositive cells were still evident.

Day 141 gestation

POMC mRNA localisation at day 141 gestation reflected the pattern found at day 134 gestation with abundant expression in the intermediate lobe and clusters of expression in the anterior lobe (Figure 5.15). Similarly, the distribution of immunoreactive melanotrophs was comparable with that found at day 134 gestation (Figure 5.16). ACTH immunopositive cells in the anterior lobe were clearly defined as single cells or clusters of cells. Palisades were no longer apparent.

5.3.2. Analysis of POMC gene expression

The mean autoradiographic grain count in the anterior and intermediate lobes of the pituitary between day 30 and day 141 gestation are shown in Figure 5.17. Due to the regional distribution of POMC mRNA in the anterior pituitary, the region around the base of the gland and the region around the intermediate lobe were analysed separately. The level of POMC mRNA in the intermediate lobe of the pituitary gland increased significantly ($P<0.05$, ANOVA) from day 50 (1.94 ± 0.24 grains/ $100\mu m^2$) to day 70 (4.39 ± 0.28 grains/ $100\mu m^2$) gestation. Levels then remained relatively constant to day 141 gestation (Figure 5.17a). In the anterior pituitary, the density of silver grains in the basal region of the gland was significntly ($p<0.01$) greater at day 40 gestation than at day 50 (1.32 ± 0.21 and 0.59 ± 0.15 grains/ $100\mu m^2$ respectively). POMC mRNA levels increased significantly ($p<0.05$) from day 50 to day 134 gestation, and then remained unchanged (Figure 5.17b). The level of POMC mRNA in the region of the anterior pituitary adjacent to the intermediate

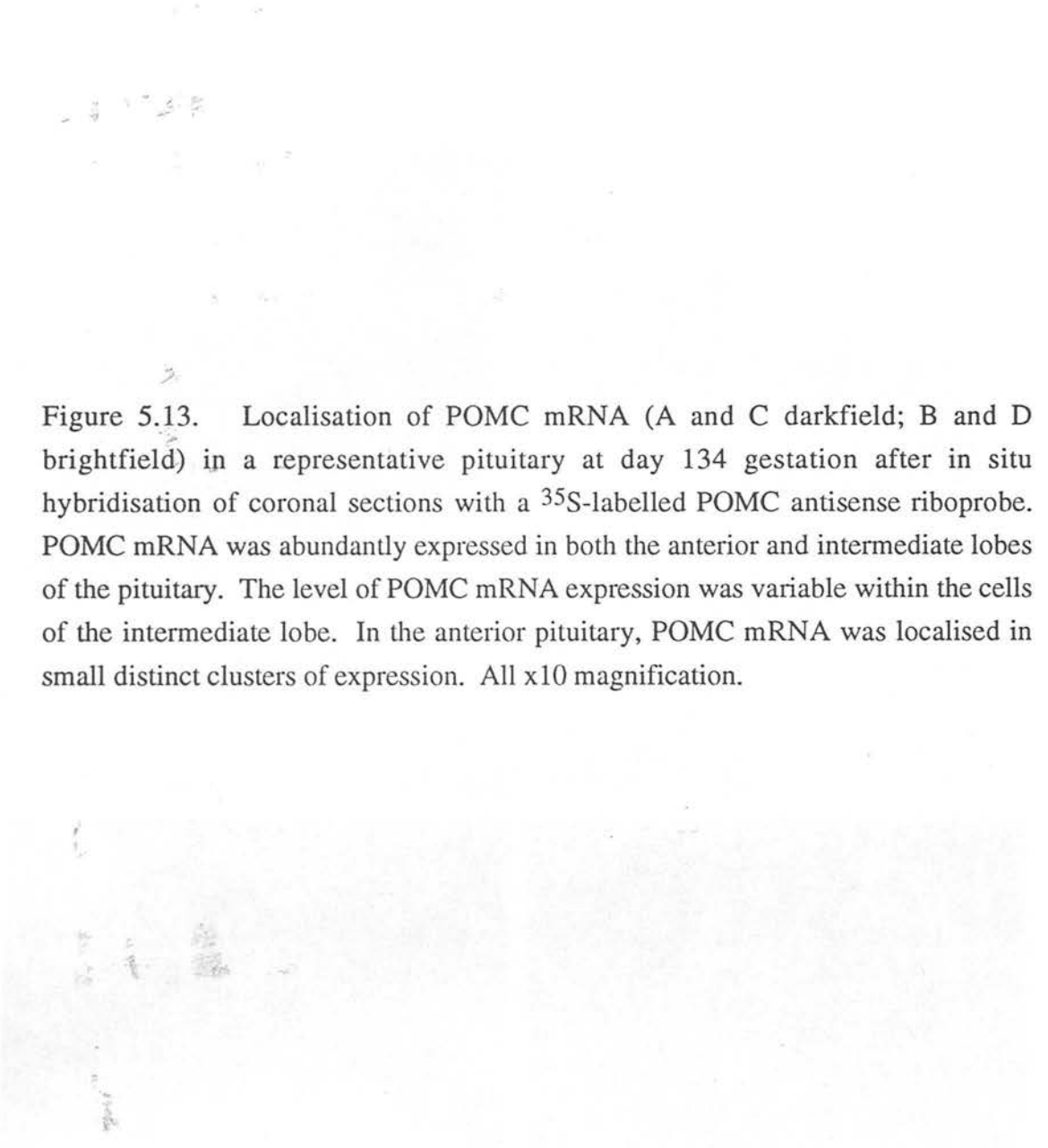


Figure 5.13. Localisation of POMC mRNA (A and C darkfield; B and D brightfield) in a representative pituitary at day 134 gestation after in situ hybridisation of coronal sections with a ^{35}S-labelled POMC antisense riboprobe. POMC mRNA was abundantly expressed in both the anterior and intermediate lobes of the pituitary. The level of POMC mRNA expression was variable within the cells of the intermediate lobe. In the anterior pituitary, POMC mRNA was localised in small distinct clusters of expression. All x10 magnification.

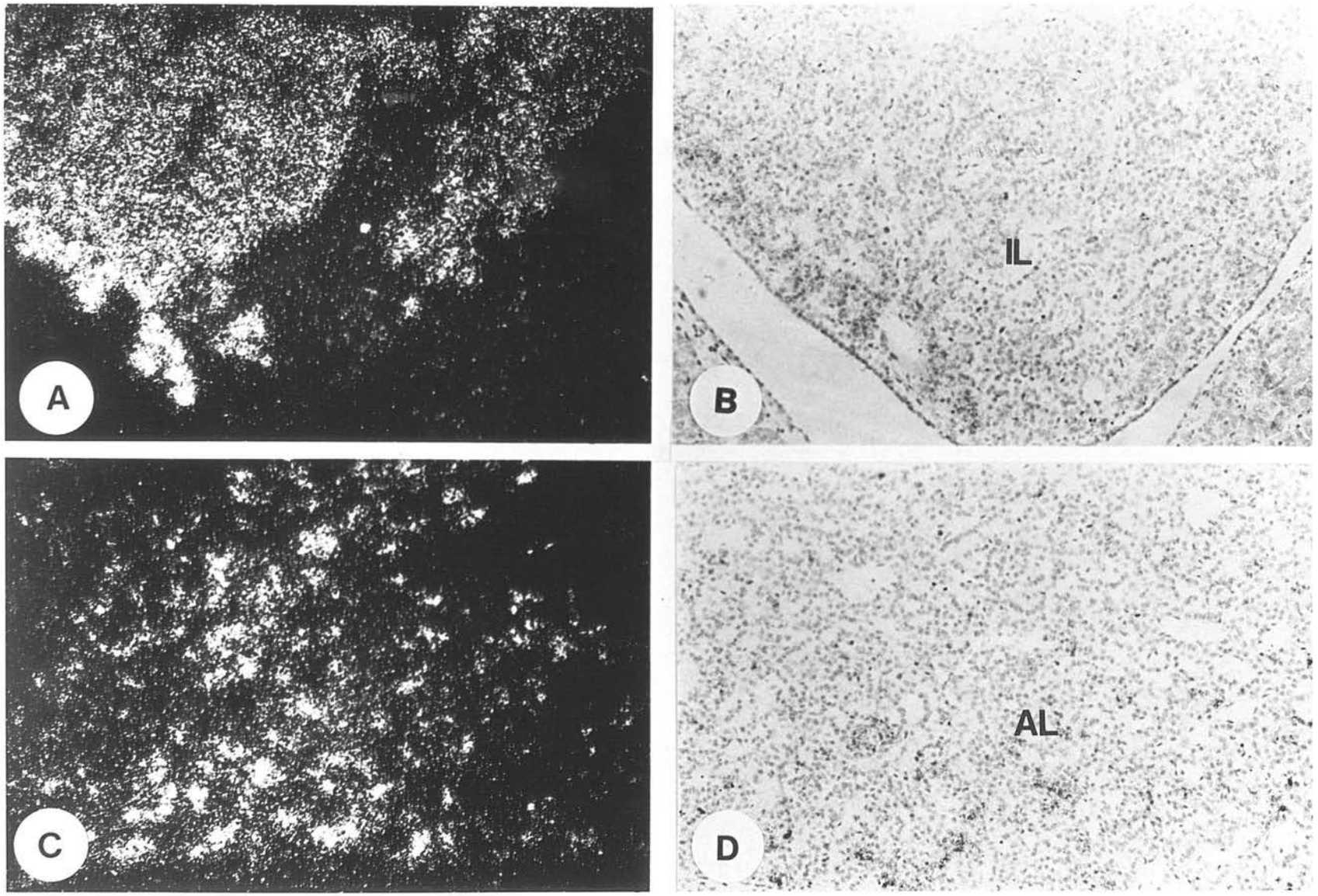

A
IL
B
C
AL
D

Figure 5.14. Immunohistochemical detection of α-MSH (A and C) and ACTH (B and D) in coronal sections of a representative pituitary at day 134 gestation. α-MSH immunostaining was confined to the intermediate lobe. ACTH was detected in both the anterior and intermediate lobes of the pituitary. Some palisades of immunopositive corticotrophs were present in the anterior pituitary. More often ACTH-immunoreactive cells were evident as individual cells or clusters with a more irregular shape. A and B x10, C x20 and D x30 magnification.

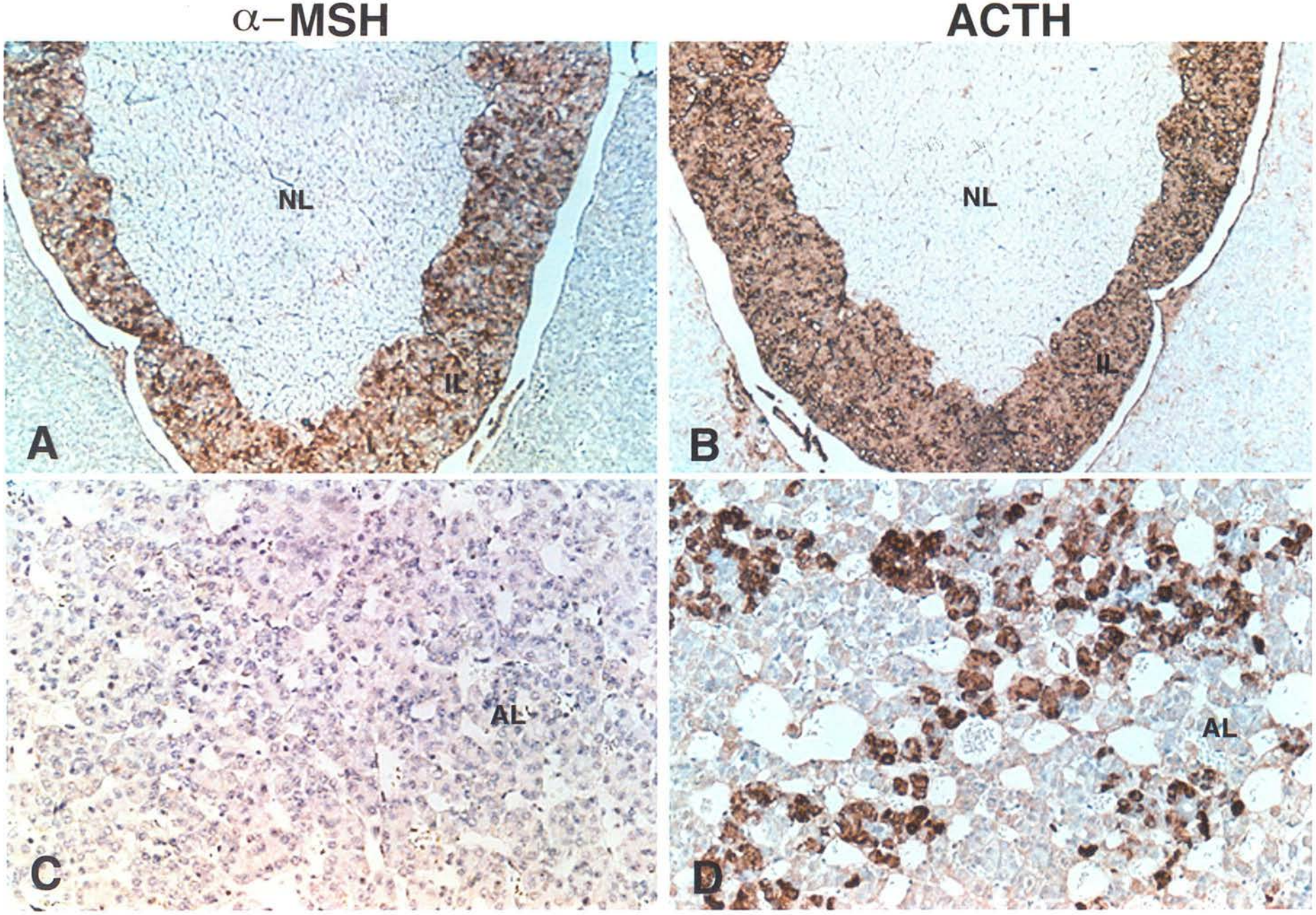

α–MSH
ACTH
NL
IL
A
NL
IL
B
AL
C
AL
D

Figure 5.15. Localisation of POMC mRNA (A and C darkfield; B and D brightfield) in a representative pituitary at day 141 gestation after in situ hybridisation of coronal sections with a ^{35}S-labelled POMC antisense riboprobe. POMC mRNA was abundantly expressed in both the anterior and intermediate lobes of the pituitary. Cellular localisation of POMC mRNA was not disimilar to that seen at day 134 gestation. All x10 magnification.

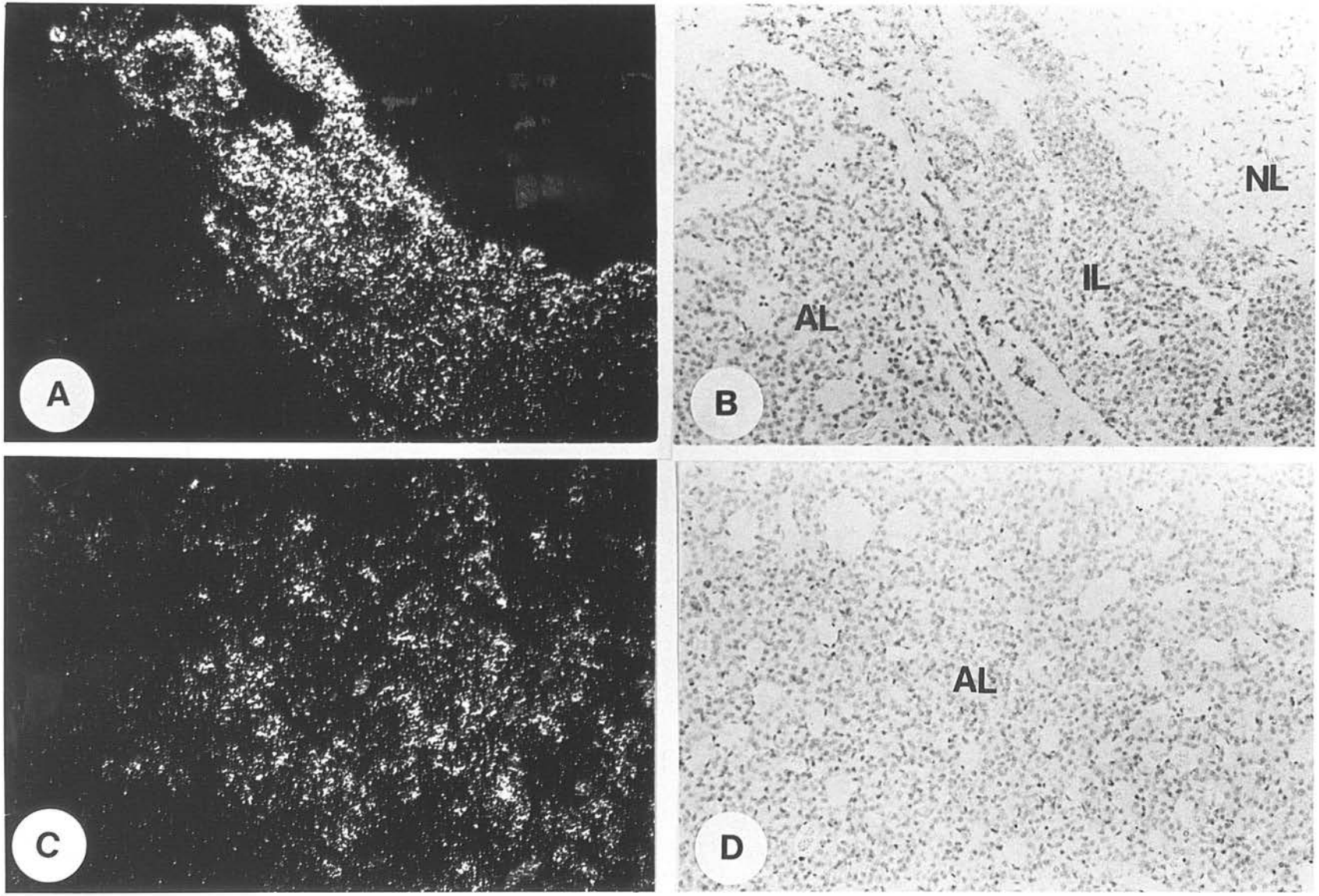
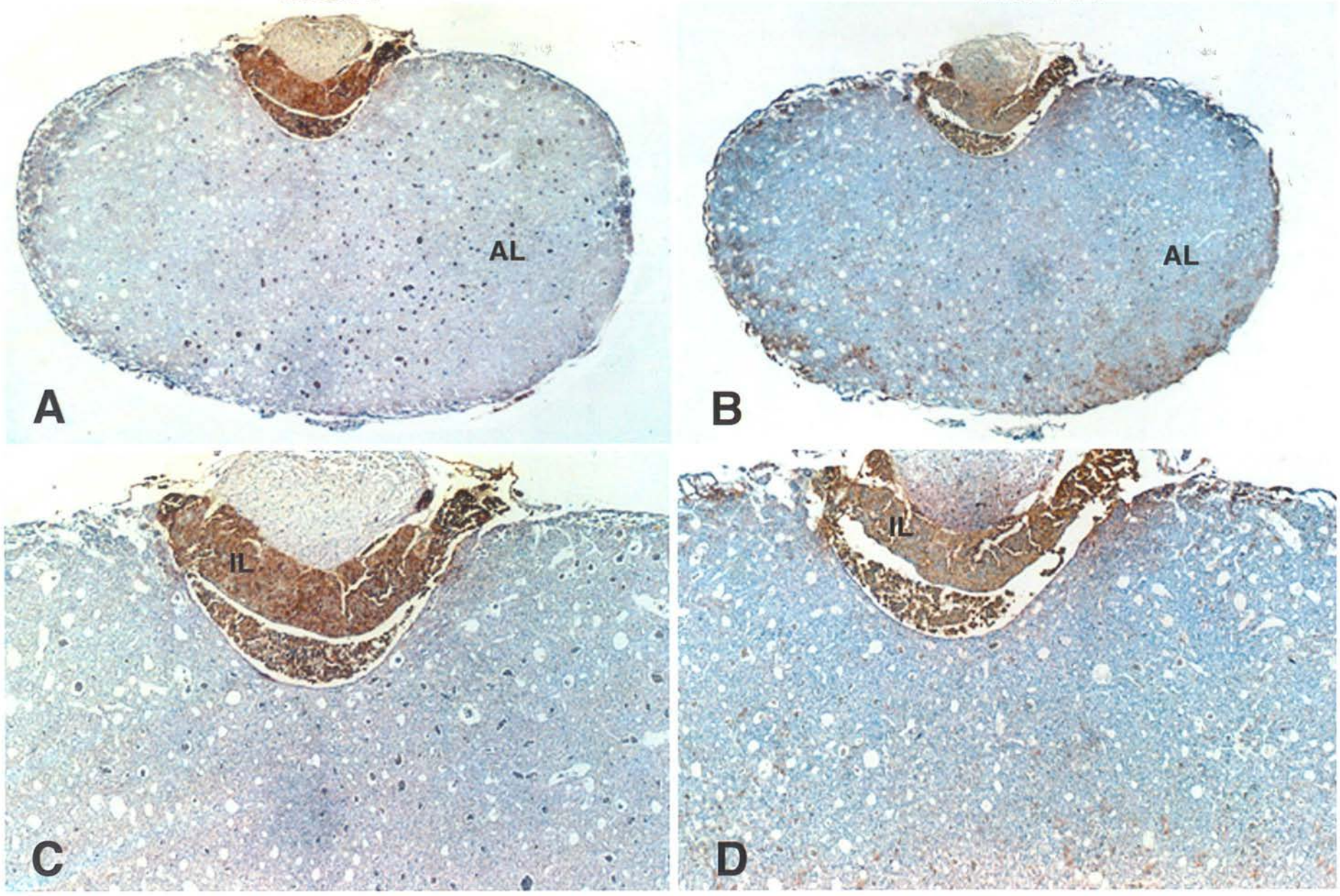

A
B
NL
IL
AL
C
D
AL

Figure 5.16. Immunohistochemical detection of α-MSH (A and C) and ACTH (B and D) in coronal sections of a representative pituitary at day 141 gestation. α-MSH immunostaining was confined to the intermediate lobe. ACTH was detected in both the anterior and intermediate lobes of the pituitary. Immunopositive corticotrophs of the anterior pituitary had a distinct irregular shape and were found as individual cells or clusters. Palisades of cells were not apparent at this gestational age. A and B x2.5, C and D x5 magnification


α–MSH
ACTH
AL
AL
A
B
IL
IL
C
D

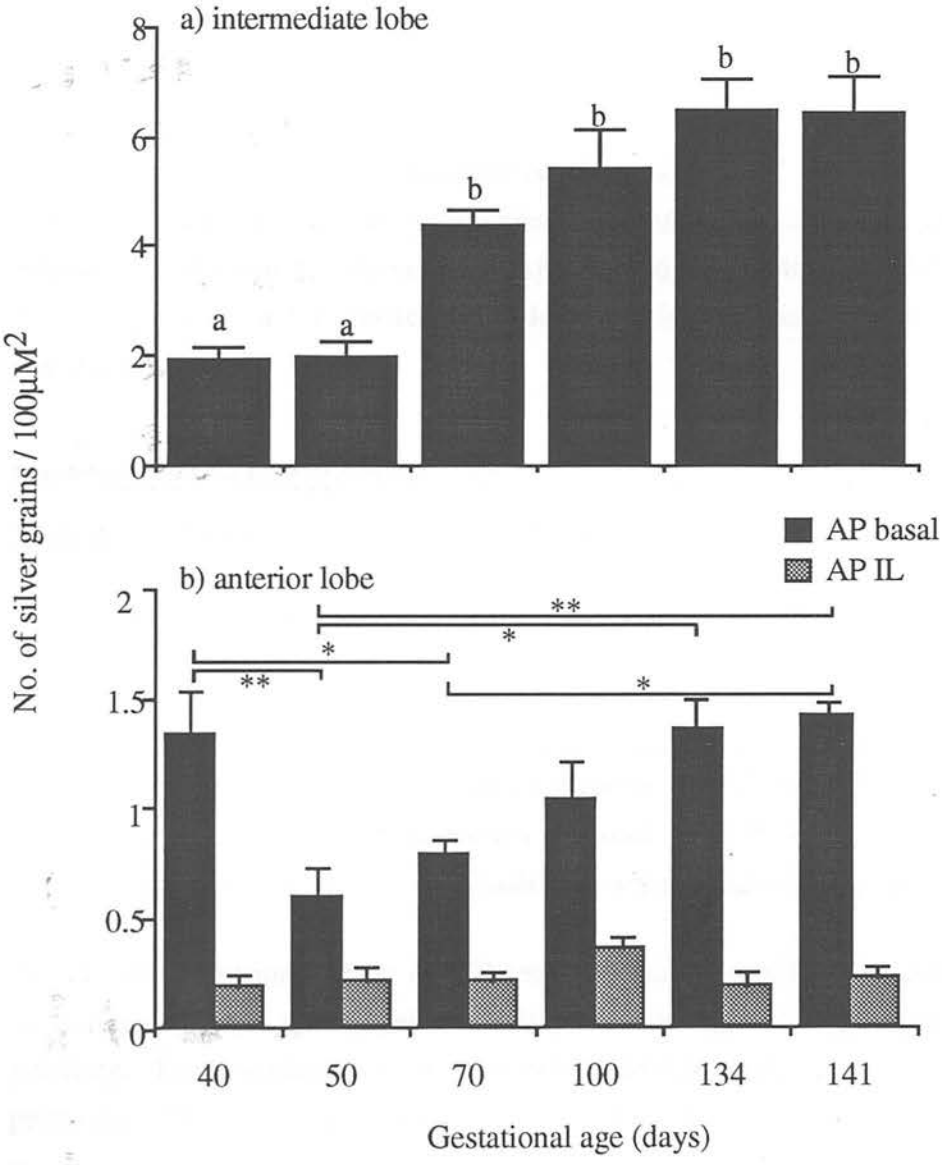


Figure 5.17. Analysis of POMC mRNA expression in the a) intermediate and b) anterior lobes of the pituitary at different gestational ages. Data shown represent the mean number of silver grains ± SEM /100µm^2. Due to regional distribution of POMC gene expression in the anterior pituitary the region around the base of the gland (black bars) and the region immediatley adjacent to the intermediate lobe (grey bars) were analysed separately. a) Values with different superscripts are significantly different (a v's b, $p<0.05$ ANOVA). b) significant differences between gestational ages are indicated by asterisks, *$p<0.05$, **$p<0.01$.

lobe was consistently lower than that found in the basal aspect of the gland and levels were unchanged with advancing gestation.

5.3.3. Percentage of immunopositive corticotrophs

Table 5.1. shows the percentage of cells immunopositive for ACTH in the anterior pituitary at different gestational ages expressed as a percentage of the total number of cells present in the corticotroph dense region of the anterior lobe at each gestational age.

Immunopositive corticotrophs (%)						
30 days	40 days	50 days	70 days	100 days	134 days	141 days
0	14.0 ± 1.7	12.4 ± 1.9	11.4 ± 0.7	14.6 ± 1.1	15.4 ± 0.8	15.3 ± 0.9

Table 5.1. Percentages of cells immunopositive for ACTH in the anterior lobe at different gestational ages. Data shown are mean ± SEM for all pituitaries at each gestational age, where the value for each pituitary represents the mean of 4 fields.

At day 40 gestation, when ACTH-immunoreactive cells were first identified, corticotrophs made up 14% of the cells present in the basal region of the anterior pituitary. The percentage of immunopositive corticotrophs fell to 11.4% at day 70 gestation. The proportion of corticotrophs then recovered to 14.6% by day 100 gestation and remained relatively constant until day 141 gestation.

5.4. Discussion

The study presented in this chapter describes the ontogeny of POMC gene expression and that of the translated POMC-derived peptides α-MSH and ACTH in the developing ovine fetal pituitary gland. POMC mRNA was first detected by in situ hybridisation at day 40 gestation in both the anterior and the intermediate lobe of the pituitary gland. The POMC-derived peptides α-MSH and ACTH were also detected at this gestational age. At all gestational ages ACTH immunoreactivity was found in both the anterior and intermediate lobes of the pituitary. In contrast, α-

MSH was confined to the intermediate lobe at most gestational ages examined, however, at day 40 and in one case at day 50 gestation, α-MSH immunopositive cells were identified in the anterior pituitary also. Ubiquitous expression of α-MSH at these early stages of development suggests that the mechanisms required for differential post-translational processing of POMC in the anterior and intermediate lobes of the pituitary are not yet functional. Post-translational processing of POMC is governed by the presence of two prohormone convertase enzymes, prohormone convertase 1 (PC1) and prohormone convertase 2 (PC2) which cleave POMC at distinct pairs of basic residues. In vitro studies revealed that co-transfection of POMC with PC1 yields peptides of the anterior pituitary, notably ACTH whilst addition of PC2 resulted in further cleavage of ACTH to produce α-MSH and CLIP (Benjannet *et al.*, 1991; Zhou *et al.*, 1993). In the adult rodent pituitary, PC1 mRNA is expressed in almost all cells of the anterior pituitary and also in some cells of the intermediate lobe (Seidah *et al.*, 1991; Day, Schafer, Watson, Chrètein and Seidah, 1992). Conversly, PC2 expression was much more abundant in the intermediate lobe (Seidah *et al.*, 1991; Day *et al.*, 1992). In contrast to the data available from the rodent, expression of PC1 and PC2 in the ovine pituitary has not been studied. The results in the present study would suggest that both PC1 and PC2 are active in the anterior lobe of the fetal pituitary early in gestation facilitating the production of α-MSH from anterior pituitary corticotrophs. This implies that tissue specific inhibition of PC2 expression must occur at a later point in development such that PC2 is no longer expressed in the anterior pituitary. Localisation of PC1 and PC2 mRNA in early gestation is needed to clarify this point. From the present study it is not possible to ascertain whether the peptides produced in the anterior lobe at day 40-50 gestation are bioactive or if they are released into the fetal circulation however, it is of interest that the hypothalamo-pituitary portal system is functional at this time (Matwijiw *et al.*, 1989; Levidiotis *et al.*, 1989). The physiological relevance of abundant anterior pituitary α-MSH expression in early gestation remains to be determined however, it is of interest that treatment of intact rat fetuses with purified anti-α-MSH inhibits both somatic growth and brain maturation (Swaab and Martin, 1981) and α-MSH restores normal fetal growth and placental development after removal of the fetal brain (Swaab and Martin, 1981). In addition, α-MSH has a more potent stimulatory effect than ACTH on DNA and protein synthesis in the fetal adrenal gland of rat, rabbit and guinea-pig in early pregnancy (Rudman *et al.*, 1980). Thus, it is interesting to speculate that the fetal anterior lobe

in early gestation acts to produce the α-MSH neeeded at this time for early fetal growth and development.

The present study represents the earliest identification of POMC mRNA in the fetal sheep pituitary gland. Previous studies have first identified POMC mRNA, both by in situ hybridisation and Northern analysis, from day 60 gestation (Yang *et al.*, 1991; McMillen *et al.*, 1988; Matthews *et al.*, 1994). However, the identification of ACTH immunoreactive cells in the anterior pituitary from as early as day 38 gestation indirectly implied the activation of the POMC gene at this time. Expression of the POMC gene during early fetal life has been demonstrated in other species (Lugo, Roberts and Pintar, 1989; Hindelang, Fèlix, Laurent, Klein and Stoeckel, 1990; Ma, Milewski, Grossman, Kato and Ellendorf, 1994). In the rat fetal pituitary, POMC mRNA is first identified in the anterior lobe followed by expression in the intermediate lobe 1-2 days later (Lugo *et al.*, 1989; Hindelang *et al.*, 1990). A similar pattern of expression has been reported in the developing pig pituitary, with POMC gene expression first detected in the anterior lobe at day 30 gestation followed by intermediate lobe expression at day 40 gestation (Ma *et al.*, 1994). In the present study, POMC mRNA was detectable in both the anterior and intermediate lobes of the pituitary at day 40 gestation. However, it is possible that the 10 day gestation period between tissue collections in the present study masks any temporal differences in anterior and intermediate pituitary expression of POMC. The concomitant appearance of POMC mRNA and immunohistochemical detection of α-MSH and ACTH in the present study agrees with data reported from the fetal rat (Hindelang *et al.*, 1989) suggesting that there is no delay between the appearance of POMC mRNA and that of translated POMC-derived products. However, the possibility remains that there is a delay in the appearance of translated POMC products in the fetal sheep pituitary following the initiation of gene expression between day 30 and day 40 gestation. This appears to be the case in the fetal pig (Ma *et al.*, 1994) in which POMC-derived peroducts are first detected 4-5 days later than POMC mRNA, suggesting that maturation of post-trancriptional processing mechanisms varies between species.

POMC mRNA levels in the basal region of the anterior pituitary were significantly greater at day 40 gestation when gene expression was first detected, than at subsequent gestational ages. The high level of POMC gene expression at day 40

gestation may reflect the augmented presence of corticotrophs over other cell types in the pituitary in early gestation suggesting that the corticotroph population in the anterior pituitary is determined early in gestation. The different cell types of the anterior pituitary arise from a common embryonic precursor in a manner which is both spatially and temporally regulated. Recently, it has become clear that tissue-specific gene expression is controlled by a variety of trans-acting factors. For lactotrophs and sommatotrophs, producing prolactin and growth hormone (GH) respectively, a single pituitary-specific transcription factor Pit-1 has been identifed and shown to activate transcription of the prolactin and growth hormone genes (Ingraham, Chan, Mangalam, Elsholtz, Flynn, Lin, Simmons, swnason and Rosenfeld, 1988). Pit-1 is not detected in corticotrophs or melanotrophs of the pituitary (Simmons, Voss, Ingraham, Holloway, Broide, Rosenfield and Swanson, 1990), both of which express the POMC gene suggesting that another factor must be responsible for cell-specific expression of POMC. The transcriptional regulation of POMC gene expression appears to be more complex than is the case for prolactin and GH, involving at least nine regulatory elements all of which act synergistically and are equally important for POMC promoter activity (Therrien and Drouin, 1991). Thus, determination of corticotrophs as a distinct cell population in the pituitary during early development requires the simultaneous action of multiple trans-acting factors.

From day 50 gestation, POMC mRNA levels in the basal region of the anterior pituitary increased progressively until day 134 gestation and then remained relatively constant. POMC mRNA levels in the region of the anterior pituitary adjacent to the intermediate lobe were consistently lower than in the rest of the anterior pituitary and levels were unchanged with advancing gestation. In the intermediate lobe, POMC mRNA levels increased significantly from day 50 to day 70 gestation. Levels continued to increase until day 134 gestation and were relatively unchanged at day 141. This data is in broad agreement with that of Matthews *et al.* (1994) who describe a similar increase in POMC mRNA levels in the intermediate lobe and basal region of the anterior lobe of the fetal sheep pituitary between day 60 and day 100 gestation. Matthews *et al.* (1994) also describe a rapid increase in POMC gene expression in the fetal anterior lobe in the days immediately preceeding parturition (day 145-147) gestation. However, this time period was not examined in the present study.

The gradual increase in POMC mRNA in the anterior pituitary described in the present study is concordant with reports of increased CRH mRNA in the paraventricular nucleus of the hypothalamus between day 100 and day 130 gestation (Myers *et al.*, 1993) and an increase in CRH secretory capacity of the fetal hypothalamus (Brooks *et al.*, 1989). These events correspond with increased concentrations of ACTH in the fetal plasma at this time (Challis and Brooks, 1989).

In agreement with the present study, Matthews *et al.* (1994) reported an increase in the proportion of ACTH-immunopositive cells in the anterior pituitary between day 60 and day 100 gestation. The present study extends this finding and suggests that earlier in gestation (day 40), corticotrophs make up a larger percentage of the total cell population present in the anterior pituitary. The fall in the proportion of corticotrophs from day 40 to day 70 gestation is likely to reflect an increase in the total number of cells present in the anterior pituitary at this time due to cell proliferation and is accompanied by a concomitant fall in the density of silver grains indicative of gene expression. Due to the marked regional distribution of corticotrophs in the anterior pituitary it was decided that determination of corticotroph number would be analysed in the corticotroph dense region at the base of the anterior pituitary. Thus, in the present study, the percentage of immunopositive corticotrophs was consistently greater than that reported by Matthews *et al.* (1994) as these workers expressed the number of corticotrophs relative to the total cell population in the entire anterior lobe.

In conclusion, the POMC gene is abundantly expressed and translated in both the intermediate and anterior lobes of the pituitary during fetal life. Levels of POMC mRNA in the anterior and intermediate lobes increase with advancing gestation and reach a constant level in late gestation. In addition, initial expression of the POMC gene in the anterior pituitary is highly abundant and is not subject to the post-translational modifications seen at later stages of development. Clearly, further studies are warranted to examine the regulation of POMC expression in early gestation.

Chapter 6. Dopaminergic regulation of pituitary POMC peptide and adrenal cortisol secretion in the ovine fetus

6.1. Introduction

Although ACTH is believed to be the main regulating hormone for the fetal adrenal cortex during the last third of gestation, discordance in the pulse characteristics of the two hormones (Apostolakis *et al.*, 1992; Brooks and Challis, 1991) and adrenal activation in the absence of increased plasma ACTH concentrations (Jacobs *et al.*, 1994) has led some to suggest that ACTH may not be solely responsible for driving cortisol secretion from the fetal adrenal in late gestation. In this respect, α-MSH, a POMC-derived peptide secreted from the intermediate lobe of the pituitary has been implicated as a trophic factor for the fetal adrenal gland and could be involved in the drive to the adrenal necessary for the prepartum cortisol surge. For instance, α-MSH has been shown to stimulate cortisol secretion both in vivo from fetal sheep, newborn lambs (Llanos *et al.*, 1979; Glickman *et al.*, 1979) and rabbits (Challis and Torosis, 1977) and from fetal sheep and human adrenal cells in culture (Glickman *et al.*, 1979). Furthermore, the stimulatory effect of α-MSH on fetal sheep adrenal cells could not be reproduced using adult cells suggesting a fetal specific role for this peptide (Glickman *et al.*, 1979). The intermediate lobe of the pituitary gland of many species is well developed during fetal life and in man diminishes progressively after birth (Visser and Swaab, 1979). POMC mRNA is abundantly expressed in the intermediate lobe of the ovine fetal pituitary (Matthews *et al.*, 1994; chapter 5) and intense immunocytochemical staining for ACTH and other POMC-derived peptides within the fetal intermediate lobe has been demonstrated (Matthews *et al.*, 1994; Mulvogue *et al.*, 1986; chapter 5). Furthermore, the lack of immunostaining for ACTH in the adult intermediate lobe suggests ontogenic differences in the processing of the POMC precursor molecule (Mulvogue *et al.*, 1986).

Evidence that the fetal intermediate lobe is under tonic dopaminergic inhibitory control comes from experiments in fetal sheep which demonstrate marked hypertrophy of the intermediate lobe after hypothalamo-pituitary disconnection (Antolovich *et al.*, 1991). During adult life, tuberohypophysial dopaminergic neurons innervate the intermediate lobe of the pituitary and inhibit intermediate lobe function via activation of dopamine D2 receptors (Björkland, Moore, Nobin and

Stevani, 1973; Munemura *et al.*, 1980a; Goudreau *et al.*, 1992; 1995; Beaulieu *et al.*, 1984; Engler, Pham, Fullerton, Clarke and Funder, 1989). However, the role of dopamine in the regulation of intermediate lobe function during fetal development is unknown.

The present study was designed to test the hypothesis that secretion of POMC-derived peptides from the ovine fetal pituitary is subject to tonic inhibitory dopaminergic regulation. Removal of this tone may release adrenal active peptides which in turn will elicit cortisol secretion from the fetal adrenal gland. Specifically, α-MSH, ACTH and cortisol responses to a 72 hour administration of either the dopamine agonist bromocriptine, the dopamine antagonist sulpiride or vehicle control in fetal sheep at day 131 gestation have been measured and effects on plasma concentrations and pulsatile characteristics of these hormones has been examined .

6.2. Materials and methods

6.2.1. Animals and surgery

27 sheep of mixed breed with known insemination dates were used in this study. Fetal catheterisation was carried out at day 125 - 126 gestation (term =145 days) as described previously in Chapter 3. After overnight recovery the ewes were moved to metabolism crates and at least 5 days were allowed before experiments began.

6.2.2. Experimental Protocol

Beginning on day 131 gestation, fetuses received an intravenous infusion of either the dopamine antagonist sulpiride (0.3mg/0.5ml/hr ; n=12,), the dopamine agonist bromocriptine (0.03mg/0.5ml/hr ; n=7) or vehicle (0.1M tartaric acid in saline ; n=8) alone. Bromocriptine, sulpiride and tartaric acid were obtained from Sigma. Blood samples (1ml) were withdrawn at 10 minute intervals for a period of four hours beginning two hours before the start of the infusion. Infusions commenced between 1100 and 1300 hours and continued for 72 hours with a second period of intensive sampling (10 minute intervals) carried out during the final 4 hours. The samples were collected in chilled heparinised tubes and centrifuged within 30 minutes in a refrigerated centrifuge at 3000rpm for 15 minutes. Plasma was stored at -20°C until assayed. Fetal and maternal blood samples were also collected twice daily (0900 and 2100hr) throughout the experimental period beginning the day before the start of

the infusion. At various times during the intensive sampling and at the time of the twice daily sampling an additional 0.2ml blood sample was withdrawn for measurement of pH, pO_2 and pCO_2 as described in Chapter 3. All fetuses included in the analysis were within the normal range; pH 7.352 ± 0.01, pO_2 20.28 ± 0.49, pCO_2 49.39 ± 0.59.

6.2.3. Measurement of plasma hormone concentrations

α-MSH and cortisol concentrations in fetal plasma were determined by radioimmunoassay and ACTH concentrations by immunoradiometric assay as described in section 3.4 and 3.5. All plasma samples were assayed in duplicate.

6.2.4. Statistical Analysis

Daily hormone concentrations were compared by Analysis of Variance (ANOVA) with repeated measures using the Abacus Concepts, Statview® package for the Apple Macintosh (Abacus Concepts, Inc., Berkeley, Ca, , 1992).

Pulse analysis was performed using the MUNRO algorithm as previously described (Martin, Taylor and McNeilly , 1987, Brooks and Challis , 1991), which detects significant excursions from a baseline followed by post-hoc t-test. A moving average of 100 minutes was used to determine the baseline value and data which increased by 2 standard deviations or more from the previous nadir were considered to be significant pulses. Data from the MUNRO pulse analysis was then subjected to two-way ANOVA on the Statview® package. All data are expressed as mean ± standard error of the mean (SEM) and values of $p \leq 0.05$ were considered to be significant.

6.3. Results

6.3.1. Changes in basal α-MSH, ACTH and cortisol concentrations over the experimental period

The concentration of α-MSH, ACTH and cortisol in fetal plasma samples collected at 12 hour intervals, beginning 24 hours prior to the start of the infusions is shown in Figure 6.1. Values shown are grouped data, such that each data point represents the mean value of all fetuses within the treatment group. There were no significant differences in the basal concentration of α-MSH, ACTH or cortisol between groups in the period before the infusions began. Infusion of the dopamine antagonist

sulpiride caused a significant ($p<0.01$) increase in plasma α-MSH concentrations 12 hours after the start of the infusion, and concentrations remained significantly elevated for the remainder of the treatment period. Plasma ACTH concentrations were also significantly ($p<0.05$) stimulated by infusion of sulpiride. The response to sulpiride was less rapid for ACTH than for α-MSH, with ACTH concentrations reaching significance 24 hours after the start of the infusion. Elevated ACTH concentrations were maintained throughout the treatment period. Treatment with the dopamine agonist bromocriptine caused a significant ($p<0.05$) and sustained decrease in concentrations of α-MSH beginning 12 hours after the start of the infusion. Bromocriptine had no effect on plasma ACTH concentrations. Cortisol concentrations were unaffected by either treatment. Additional analysis confirmed that cortisol concentrations following 72 hour infusion of bromocriptine or sulpiride were not significantly different from vehicle treated controls (Mann-Whitney U test; $p= 0.99$ and $p= 0.44$ respectively).

6.3.2. Acute hormone responses to treatment with bromocriptine or sulpiride

Figure 6.2 shows plasma α-MSH concentrations in a representative fetus from each treatment group during the course of the first 4 hour period of intensive blood sampling. There was a rapid increase in α-MSH secretion to the fetal plasma with significantly increased levels of α-MSH apparent in the fetal circulation within 10 minutes of the start of the sulpiride infusion. The response to bromocriptine is less rapid with plasma α-MSH concentrations steadily declining throughout the duration of the sampling period. In contrast to the changes in circulating α-MSH concentration, plasma concentrations of ACTH (Figure 6.3) and cortisol (Figure 6.4) were not acutely influenced by treatment with either bromocriptine or sulpiride.

The profiles of α-MSH, ACTH and cortisol secretion obtained during the period of intensive sampling, of which figures 6.2 to 6.4 are representative, were further analysed in terms of pulsatile characteristics. Data shown in figures 6.5 to 6.7 are grouped values representing the mean value for all fetuses within each treatment group.

The Munro pulse analysis program successfully detected pulses of α-MSH, ACTH and cortisol in all fetuses examined. There were no changes in either the frequency, amplitude or mean hormone concentrations of any of the hormones measured in response to vehicle alone. Mean plasma α-MSH concentration, pulse amplitude and

Figure 6.1. α-MSH, ACTH and cortisol concentrations in fetal plasma samples collected at 12 hour intervals over a four day period from day 130 to day 134 gestation. Intravenous infusion of vehicle (n=8), bromocriptine (n=7) or sulpiride (n=12) began after 24 hours and continued for 72 hours. Data shown are the mean values ± SEM for all fetuses within each treatment group . Significant differences are represented by a) $p<0.05$ and b) $p<0.01$ from vehicle control.

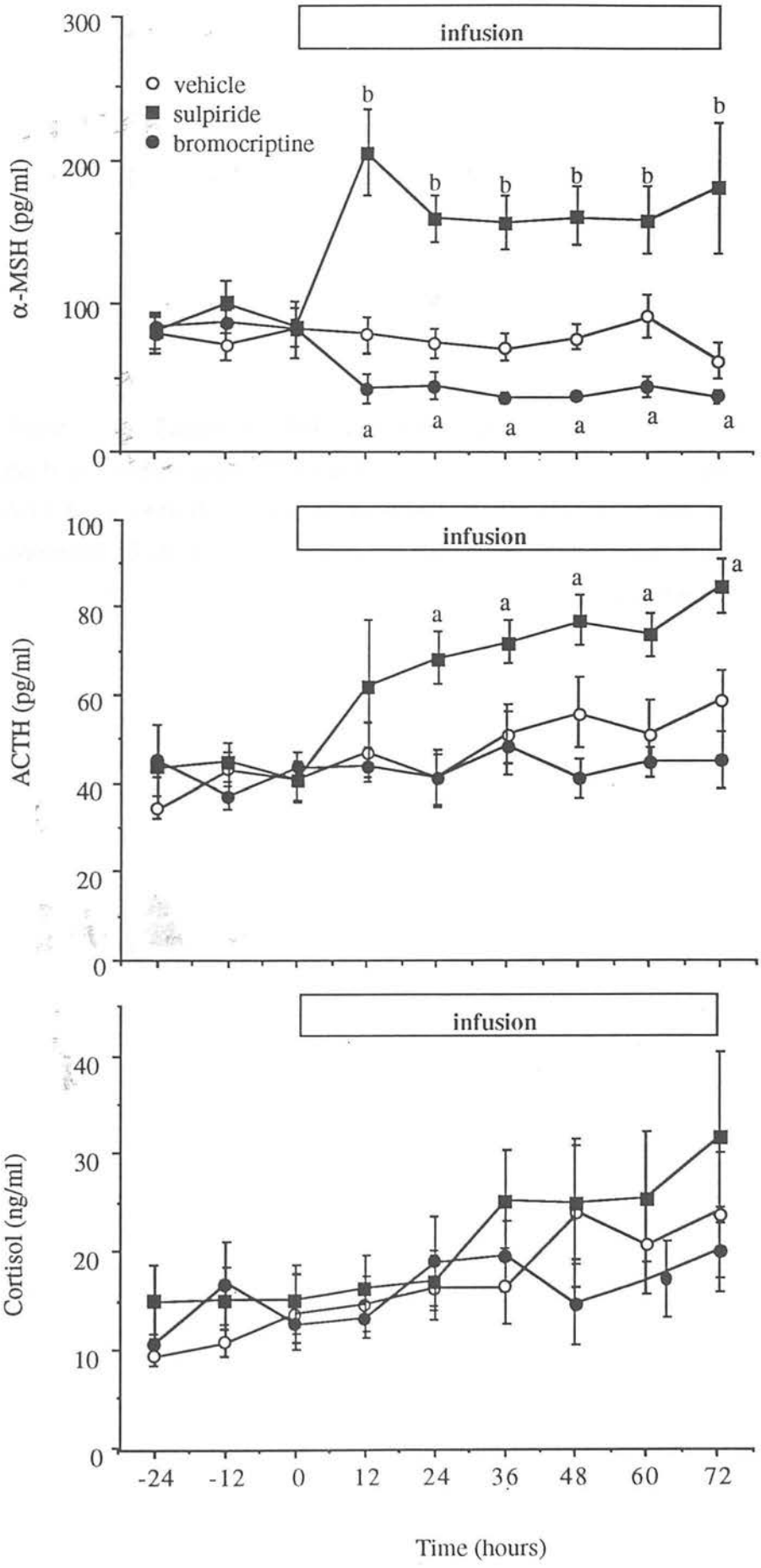

infusion
vehicle
sulpiride
bromocriptine
α-MSH (pg/ml)
300
200
100
0
b
a
infusion
ACTH (pg/ml)
100
80
60
40
20
0
infusion
Cortisol (ng/ml)
40
30
20
10
0
-24
-12
0
12
24
36
48
60
72
Time (hours)

Figure 6.2. Plasma α-MSH concentrations in 3 representative fetuses, one from each treatment group. Plasma samples were collected at 10 minute intervals over a four hour period. Intravenous infusion of vehicle, bromocriptine or sulpiride commenced after the first 2 hours of sampling. Significant pulses identified by the MUNRO pulse analysis program are represented by an arrowhead.

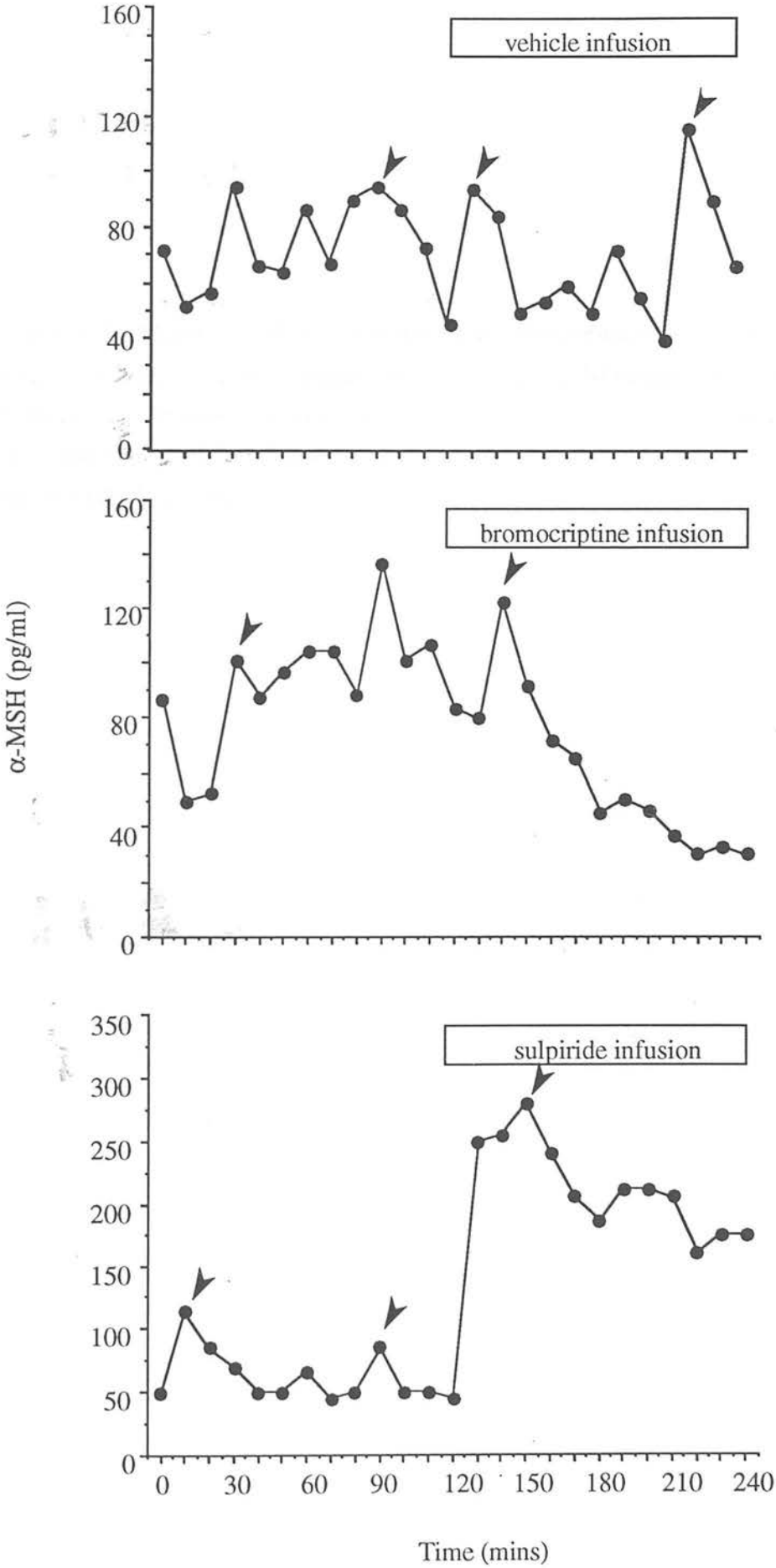

vehicle infusion
bromocriptine infusion
sulpiride infusion
α-MSH (pg/ml)
Time (mins)
0
40
80
120
160
50
100
150
200
250
300
350
0
30
60
90
120
150
180
210
240

Figure 6.3. Plasma ACTH concentrations in 3 representative fetuses, one from each treatment group. Plasma samples were collected at 10 minute intervals over a four hour period. Intravenous infusion of vehicle, bromocriptine or sulpiride commenced after the first 2 hours of sampling. Significant pulses identified by the MUNRO pulse analysis program are represented by an arrowhead.

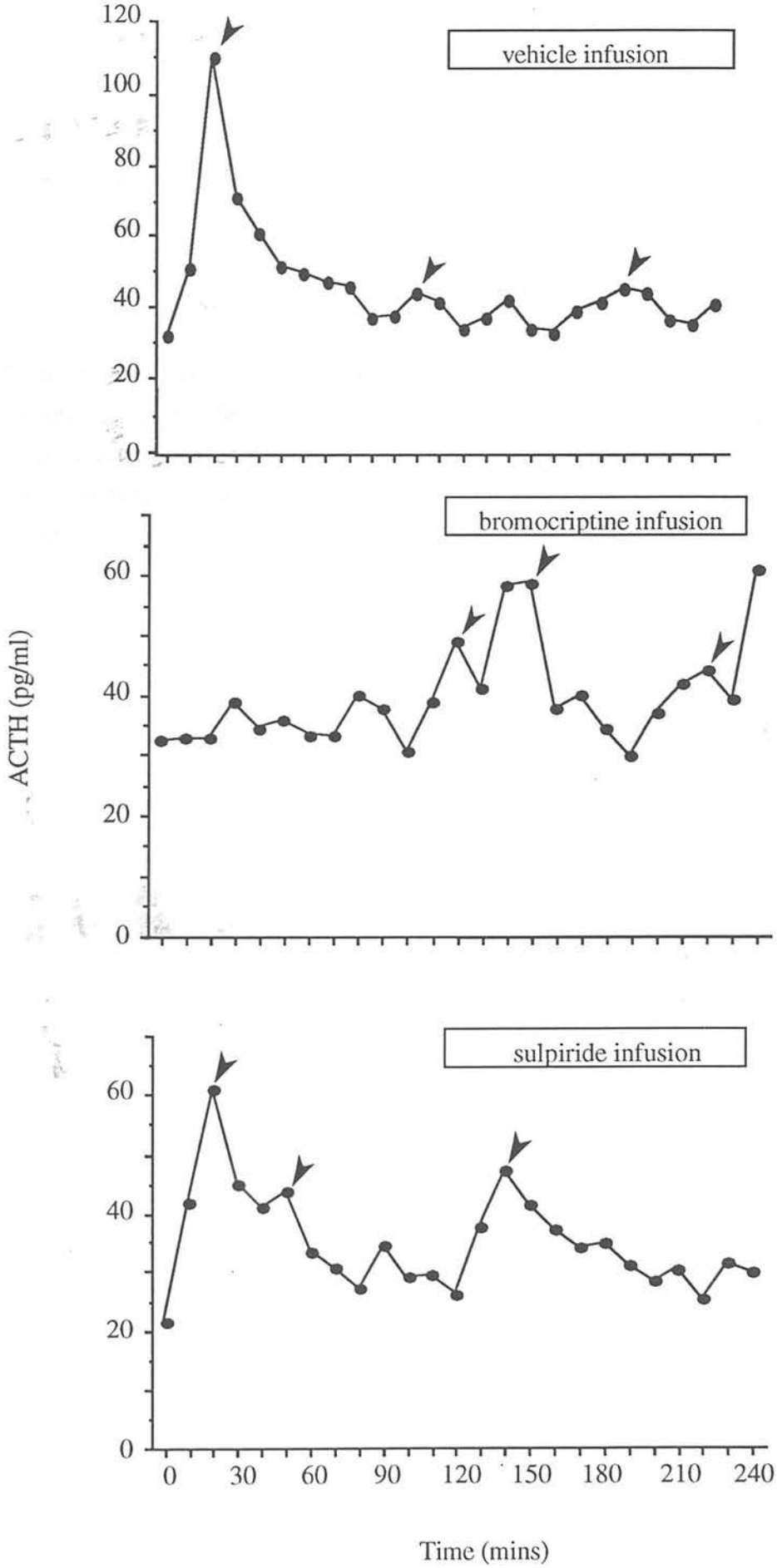

vehicle infusion
bromocriptine infusion
sulpiride infusion
ACTH (pg/ml)
Time (mins)
0
20
40
60
80
100
120
0
30
60
90
120
150
180
210
240

Figure 6.4. Plasma cortisol concentrations in 3 representative fetuses, one from each treatment group. Plasma samples were collected at 10 minute intervals over a four hour period. Intravenous infusion of vehicle, bromocriptine or sulpiride commenced after the first 2 hours of sampling. Significant pulses identified by the MUNRO pulse analysis program are represented by an arrowhead.

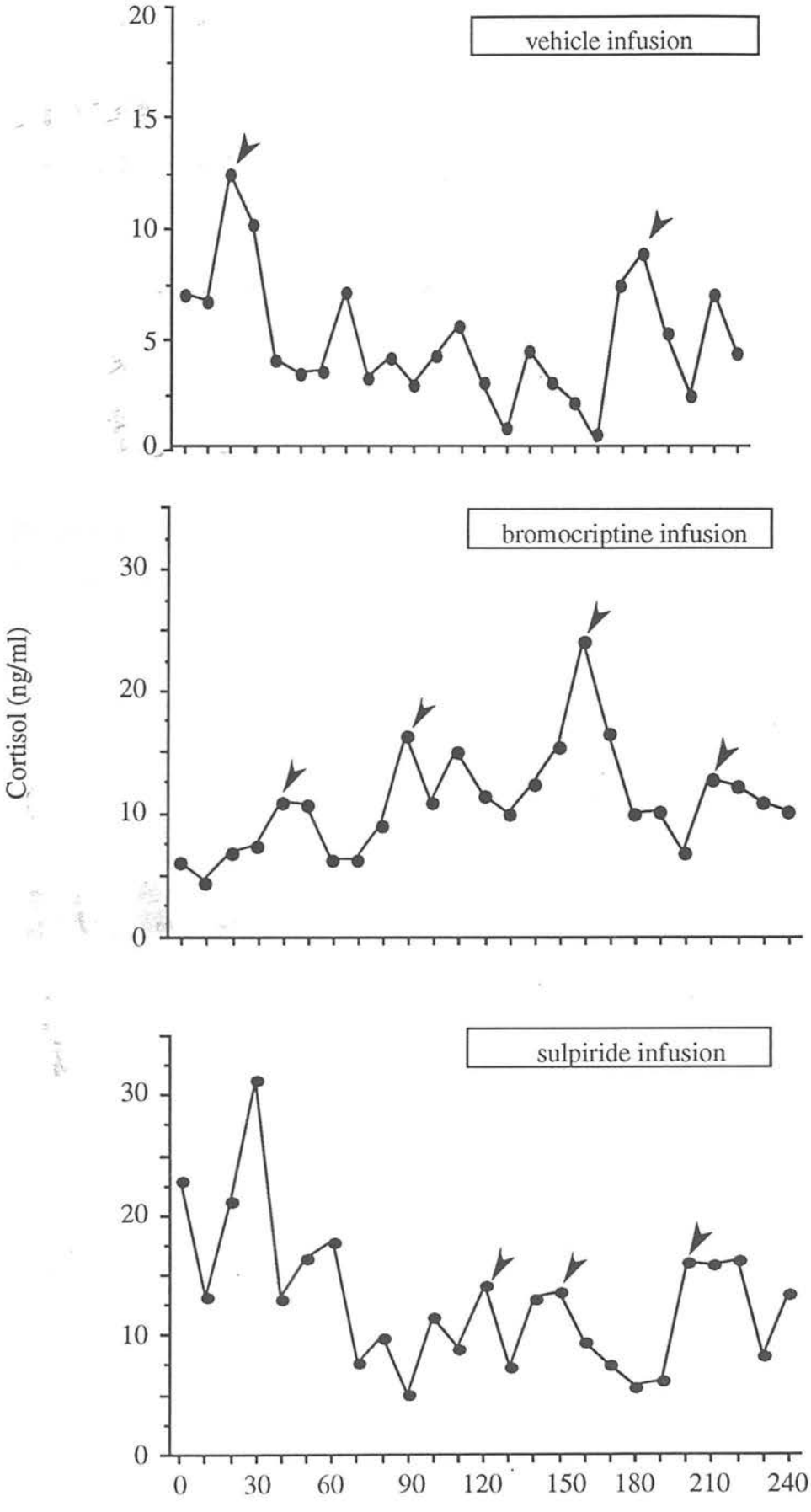

vehicle infusion
bromocriptine infusion
sulpiride infusion
Cortisol (ng/ml)
Time (mins)
0
5
10
15
20
30
0
30
60
90
120
150
180
210
240

Figure 6.5. Analysis of pulse characteristics of α-MSH secretion comparing the 2 hour basal period with the first 2 hours of vehicle (n=8), bromocriptine (n=7) or sulpiride (n=12) infusion. Data shown are means ± SEM of all fetuses in each treatment group. Significant differences compared with corresponding controls are indicated by asterisks, * $p<0.05$, *** $p<0.001$.

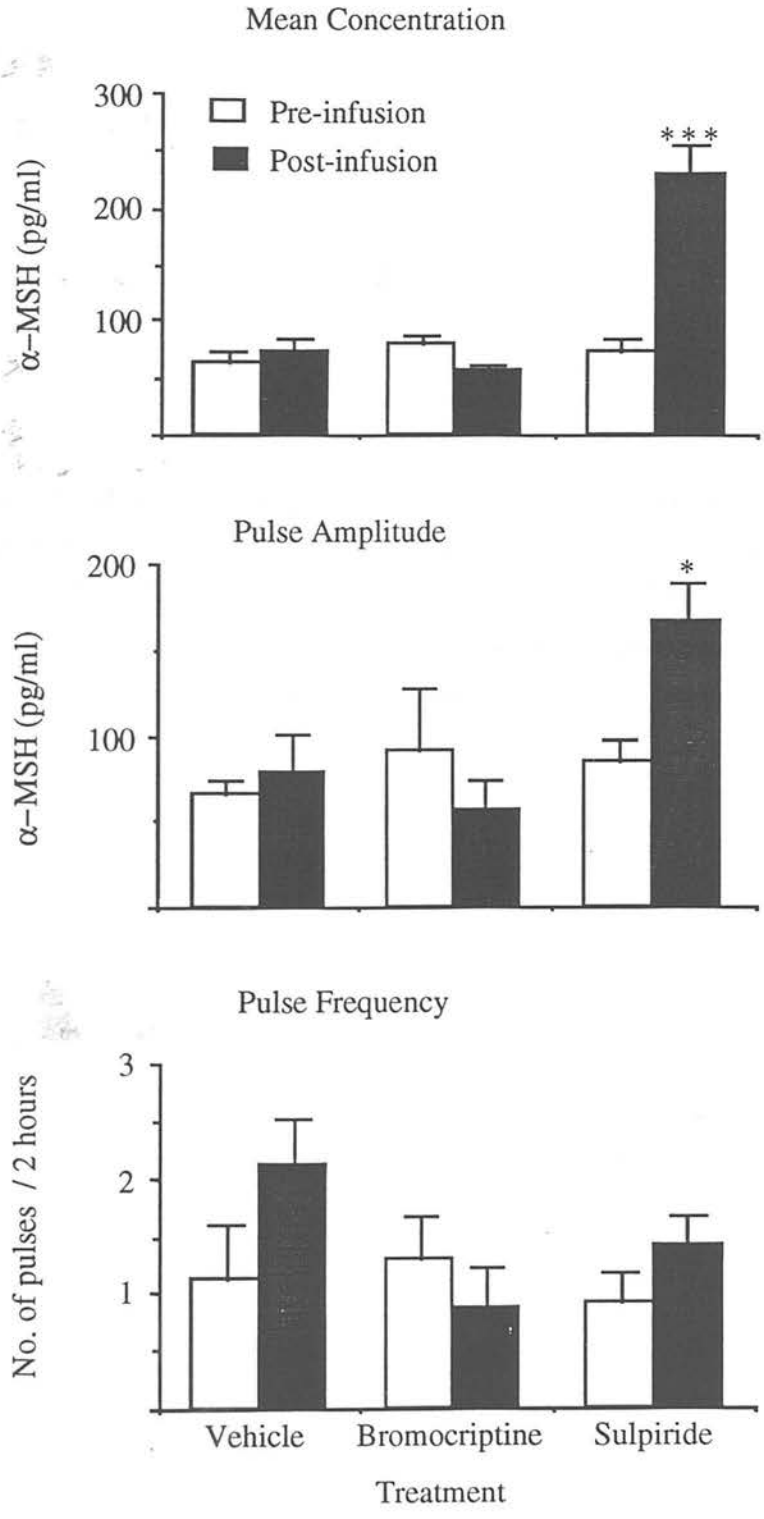

Mean Concentration
300
200
100
α–MSH (pg/ml)
Pre-infusion
Post-infusion
***
Pulse Amplitude
200
100
α–MSH (pg/ml)
*
Pulse Frequency
3
2
1
No. of pulses / 2 hours
Vehicle
Bromocriptine
Sulpiride
Treatment

Figure 6.6. Analysis of pulse characteristics of ACTH secretion comparing the 2 hour basal period with the first 2 hours of vehicle (n=8), bromocriptine (n=7) or sulpiride (n=12) infusion. Data shown are means ± SEM of all fetuses in each treatment group. There were no significant differences between treated and control groups for any of the measured characteristics.

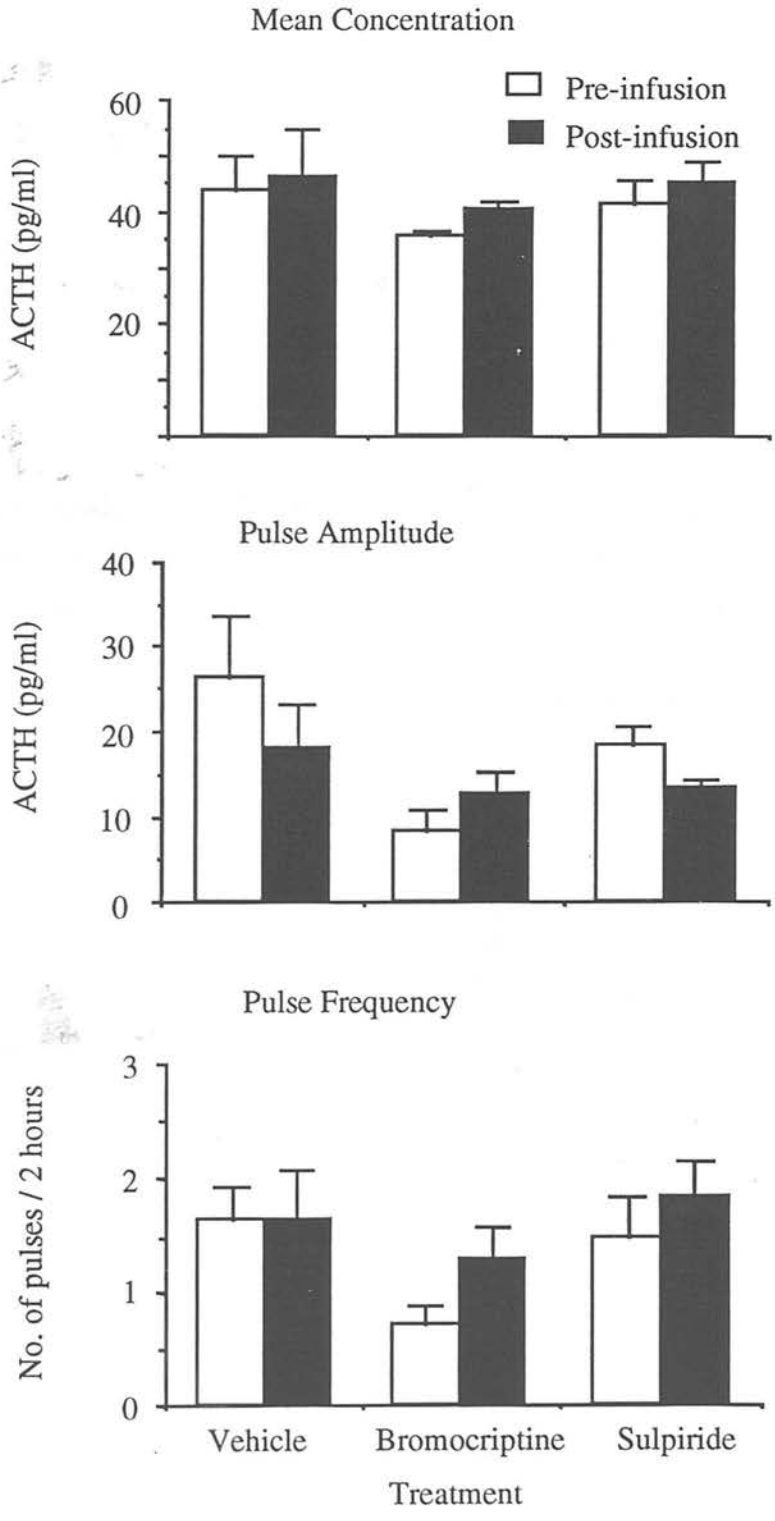
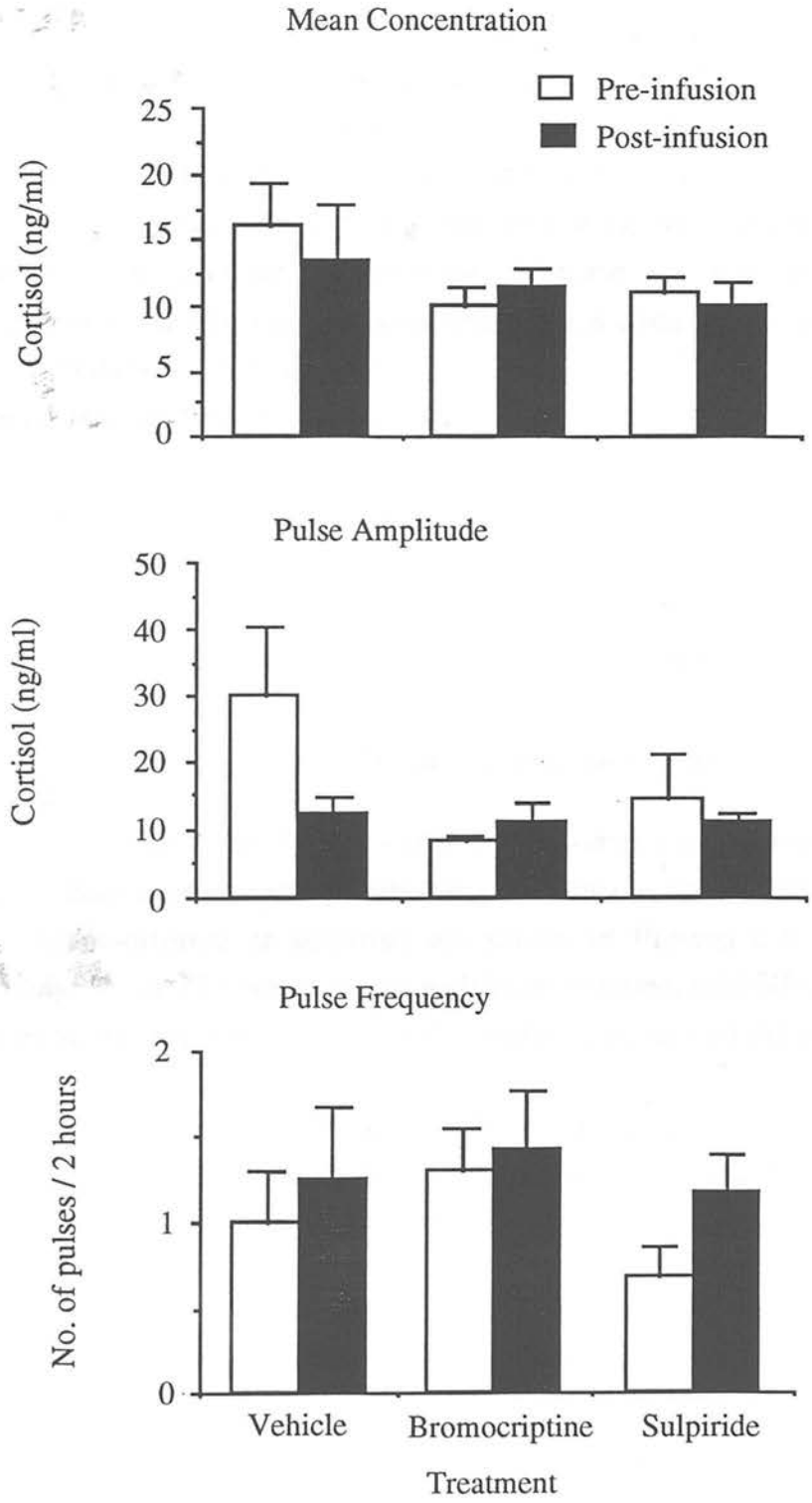

Mean Concentration
Pre-infusion
Post-infusion
ACTH (pg/ml)
60
40
20
Pulse Amplitude
ACTH (pg/ml)
40
30
20
10
0
Pulse Frequency
No. of pulses / 2 hours
3
2
1
0
Vehicle
Bromocriptine
Sulpiride
Treatment

Figure 6.7. Analysis of pulse characteristics of cortisol secretion comparing the 2 hour basal period with 2 hours of vehicle (n=8), bromocriptine (n=7) or sulpiride (n=12) infusion. Data shown are means of all fetuses in each treatment group ± SEM. There was no significant effect of any treatment on mean measured plasma ACTH concentration, ACTH pulse amplitude or pulse frequency.


Mean Concentration
Pre-infusion
Post-infusion
Cortisol (ng/ml)
25
20
15
10
5
0
Pulse Amplitude
Cortisol (ng/ml)
50
40
30
20
10
0
Pulse Frequency
No. of pulses / 2 hours
2
1
0
Vehicle
Bromocriptine
Sulpiride
Treatment

pulse frequency in the two hour period before the infusion and the two hour period following the start of the infusion are shown in Figure 6.5. The mean plasma concentration of α-MSH increased significantly ($P<0.001$) in the 2 hour post-infusion period in sulpiride-treated fetuses when compared to the 2 hour period prior to the start of the infusion. This increase in mean plasma α-MSH concentration was accompanied by a significant ($P<0.05$) increase in α-MSH pulse amplitude during this period. α-MSH pulse frequency was unaffected by sulpiride treatment. The steady decline in α-MSH levels during the course of the bromocriptine infusion was not sufficient to produce a significant decrease in mean plasma α-MSH concentration in the two hour infusion period when compared to the pre-infusion period. Similarly, bromocriptine treatment had no significant effect on either α-MSH pulse frequency or pulse amplitude.

Examination of the pulsatile characteristics of ACTH (Figure 6.6) and cortisol (Figure 6.7) secretion in response to the two hour infusion of either bromocriptine or sulpiride revealed that neither treatment had any significant effect on the mean plasma concentration, pulse amplitude or pulse frequency of these hormones.

6.3.3. Hormone responses after 72 hours of treatment with bromocriptine or sulpiride

The profile of α-MSH, ACTH and cortisol secretion in a representative fetus from each of the three treatment groups following a continuous 72 hour infusion, of either vehicle, bromocriptine or sulpiride are shown in Figures 6.8, 6.9 and 6.10 respectively. After 72 hour treatment with bromocriptine, α-MSH was suppressed to concentrations that were at or below the level of sensitivity of the assay.

There were no changes in plasma ACTH or cortisol concentrations following bromocriptine infusion. In contrast, sulpiride treatment significantly increased the plasma concentration of both α-MSH and ACTH when compared to vehicle treated controls.

The pulse profiles of individual fetuses obtained during the second intensive sampling period were analysed using the MUNRO pulse analysis program. Grouped data for mean plasma α-MSH concentration, pulse amplitude and pulse frequency for all fetuses in each treatment group following the 72 hour infusion period are shown in Figure 6.11. 72 hour infusion of sulpiride resulted in an increase in basal

secretion of α-MSH, such that the mean concentration of α-MSH was significantly ($P<0.01$) greater in sulpiride-treated fetuses compared to vehicle-treated control fetuses over the four hour period. Sulpiride-treated fetuses also displayed a significant ($P<0.001$) increase in the amplitude of α-MSH pulses, together with a significant ($p<0.01$) decrease in pulse frequency. Following bromocriptine infusion there was a significant ($p<0.001$) decrease in mean plasma α-MSH concentration and pulse frequency when compared to vehicle-treated controls. The decrease in pulse frequency was such that only 1 pulse was detected in each of two fetuses (i.e. a total of 2 pulses) and so it was not possible to compare the amplitude of these pulses statistically. The mean amplitude for these two pulses is shown in Figure 6.11.

ACTH pulse characteristics are shown in Figure 6.12. The mean ACTH concentrations in sulpiride-treated fetuses were significantly ($p<0.001$) higher than in control fetuses. ACTH pulse amplitude and pulse frequency were unaffected by infusion of either bromocriptine or sulpiride.

72 hour infusion of bromocriptine or sulpiride did not significantly alter the mean plasma cortisol concentration, cortisol pulse amplitude or pulse frequency (Figure 6.13) when compared to vehicle-treated controls.

6.4. Discussion

The results presented in this chapter demonstrate that secretion of α-MSH and ACTH from the fetal pituitary gland is regulated by dopamine. The secretion of α-MSH from the fetal pituitary gland is significantly increased by intravenous infusion of the dopamine antagonist sulpiride and decreased by infusion of the dopamine agonist bromocriptine, whilst, the secretion of ACTH is increased by sulpiride but unaffected by bromocriptine. Despite these changes in α-MSH and ACTH there were no significant changes in fetal plasma cortisol concentrations.

The results presented in this chapter represent the first identification of pulsatile secretion of α-MSH from the fetal pituitary gland. Secretion of ACTH and α-MSH into the circulation of adult sheep has been investigated using a rapid blood sampling procedure (Engler *et al.*, 1989). In this study, blood samples were collected from conscious adult sheep at 10 minute intervals for 4 hours and in another group at 2 minute intervals for 1 hour. The authors demonstrated the

Figure 6.8. Plasma α-MSH concentrations in 3 representative fetuses, one from each treatment group. Blood samples were collected at 10 minute intervals over a 4 hour period at the end of a 72 hour infusion vehicle, bromocriptine or sulpiride. Significant pulses identified by the MUNRO pulse analysis program are represented by an arrowhead.

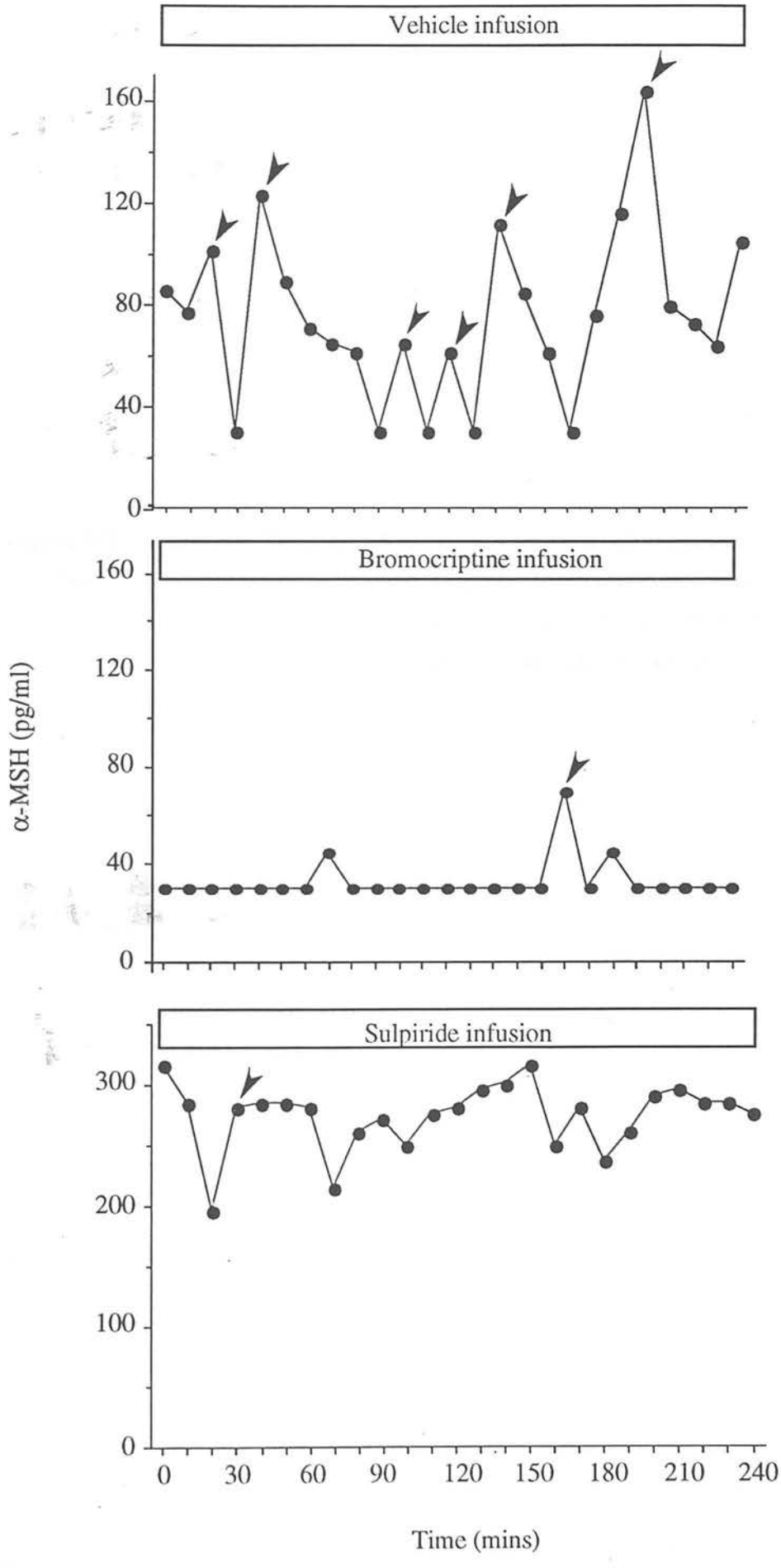

Vehicle infusion
160
120
80
40
0
Bromocriptine infusion
160
120
80
40
0
Sulpiride infusion
300
200
100
0
α-MSH (pg/ml)
0
30
60
90
120
150
180
210
240
Time (mins)

Figure 6.9. Plasma ACTH concentrations in 3 representative fetuses, one from each treatment group. Blood samples were collected at 10 minute intervals over a 4 hour period at the end of a 72 hour infusion vehicle, bromocriptine or sulpiride. Significant pulses identified by the MUNRO pulse analysis program are represented by an arrowhead.

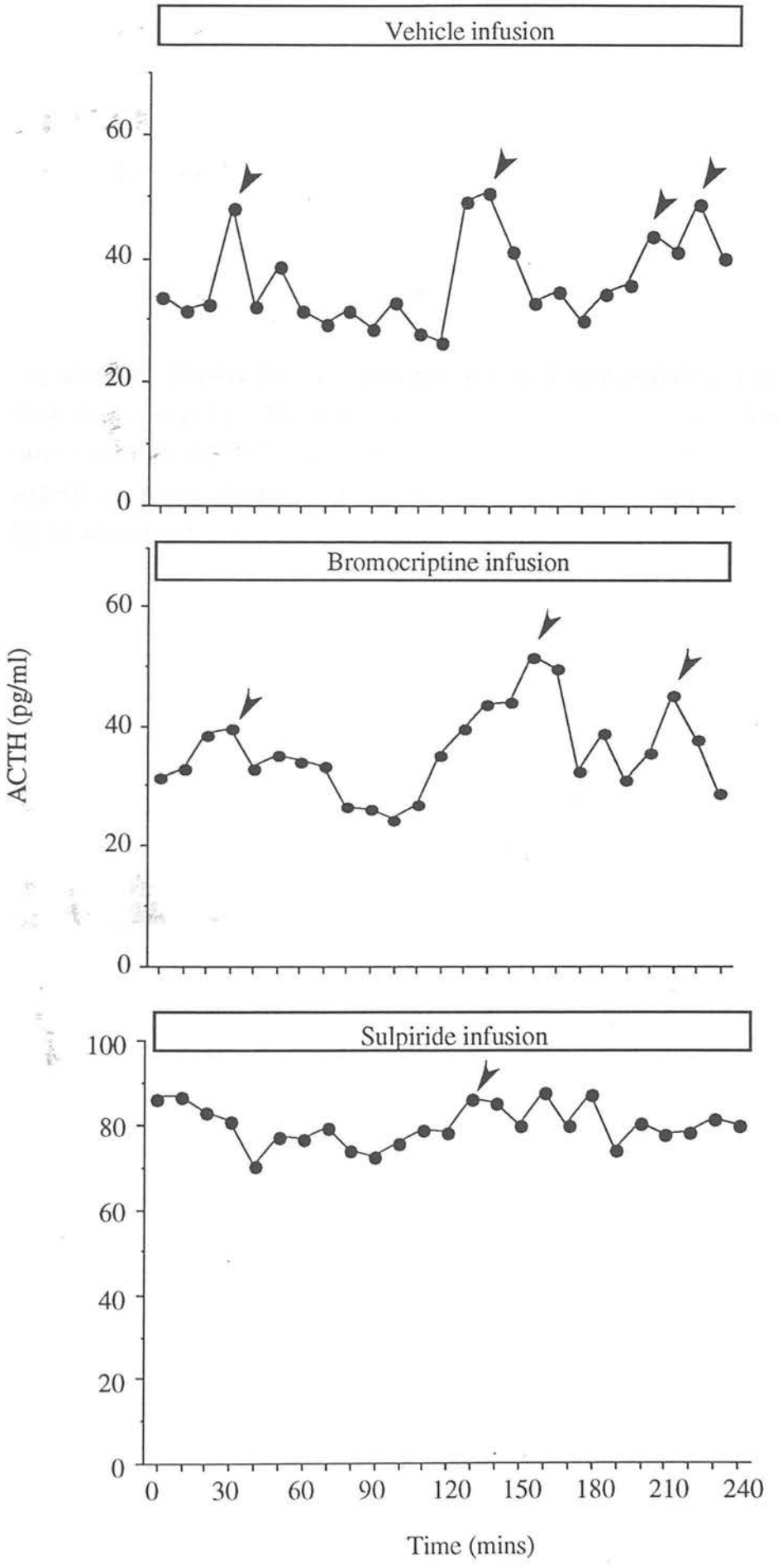

Vehicle infusion
Bromocriptine infusion
Sulpiride infusion
ACTH (pg/ml)
0
20
40
60
80
100
0
30
60
90
120
150
180
210
240
Time (mins)

Figure 6.10. Plasma cortisol concentrations in 3 representative fetuses, one from each treatment group. Blood samples were collected at 10 minute intervals over a 4 hour period at the end of a 72 hour infusion vehicle, bromocriptine or sulpiride. Significant pulses identified by the MUNRO pulse analysis program are represented by an arrowhead.

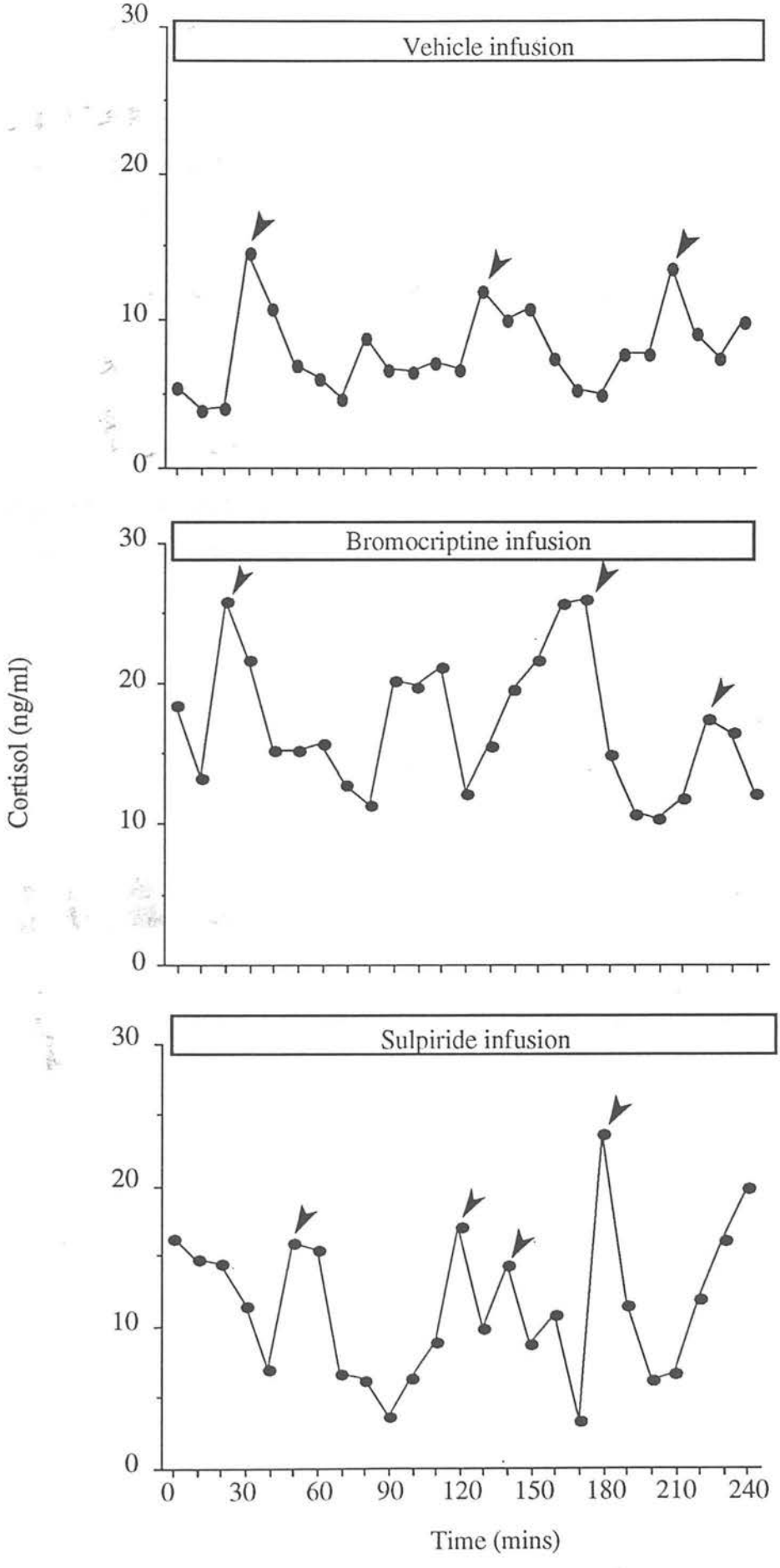

Vehicle infusion
Bromocriptine infusion
Sulpiride infusion
Cortisol (ng/ml)
0
10
20
30
0
30
60
90
120
150
180
210
240
Time (mins)

Figure 6.11. Analysis of α-MSH pulse characteristics following 72 hour infusion of vehicle (n=8), bromocriptine (n=7) or sulpiride (n=12). Data shown are means ± SEM of all fetuses in each treatment group. Significant differences compared with corresponding controls are indicated by asterisks, ** $p<0.01$, *** $p<0.001$.

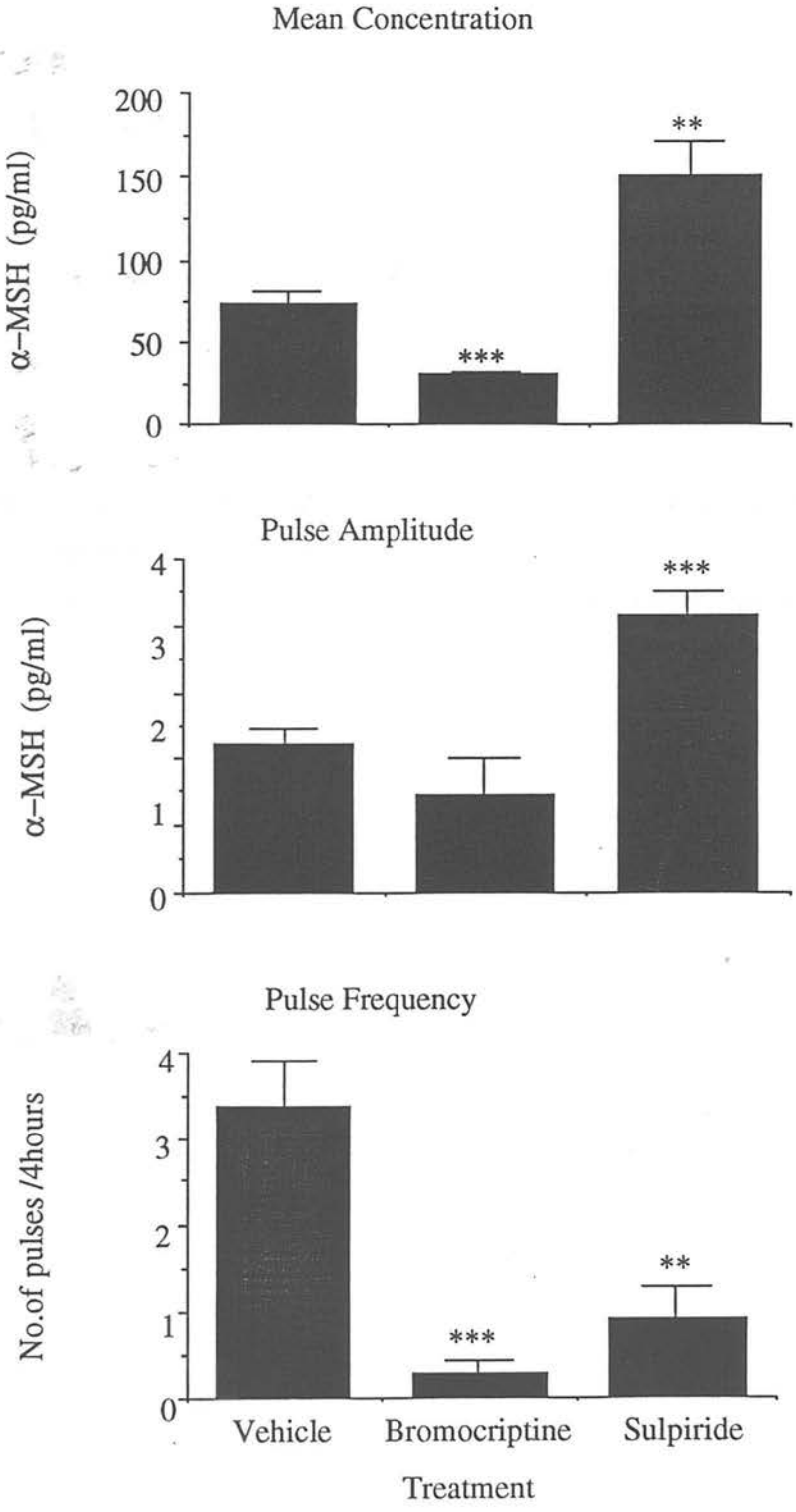

Mean Concentration
α–MSH (pg/ml)
200
150
100
50
0
**
***
Pulse Amplitude
α–MSH (pg/ml)
4
3
2
1
0
***
Pulse Frequency
No.of pulses /4hours
4
3
2
1
0
***
**
Vehicle
Bromocriptine
Sulpiride
Treatment

Figure 6.12. Analysis of ACTH pulse characteristics following 72 hour infusion of vehicle (n=8), bromocriptine (n=7) or sulpiride (n=12). Data shown are means ± SEM of all fetuses in each treatment group. Significant differences compared with corresponding controls are indicated by asterisks, *** $p<0.001$.

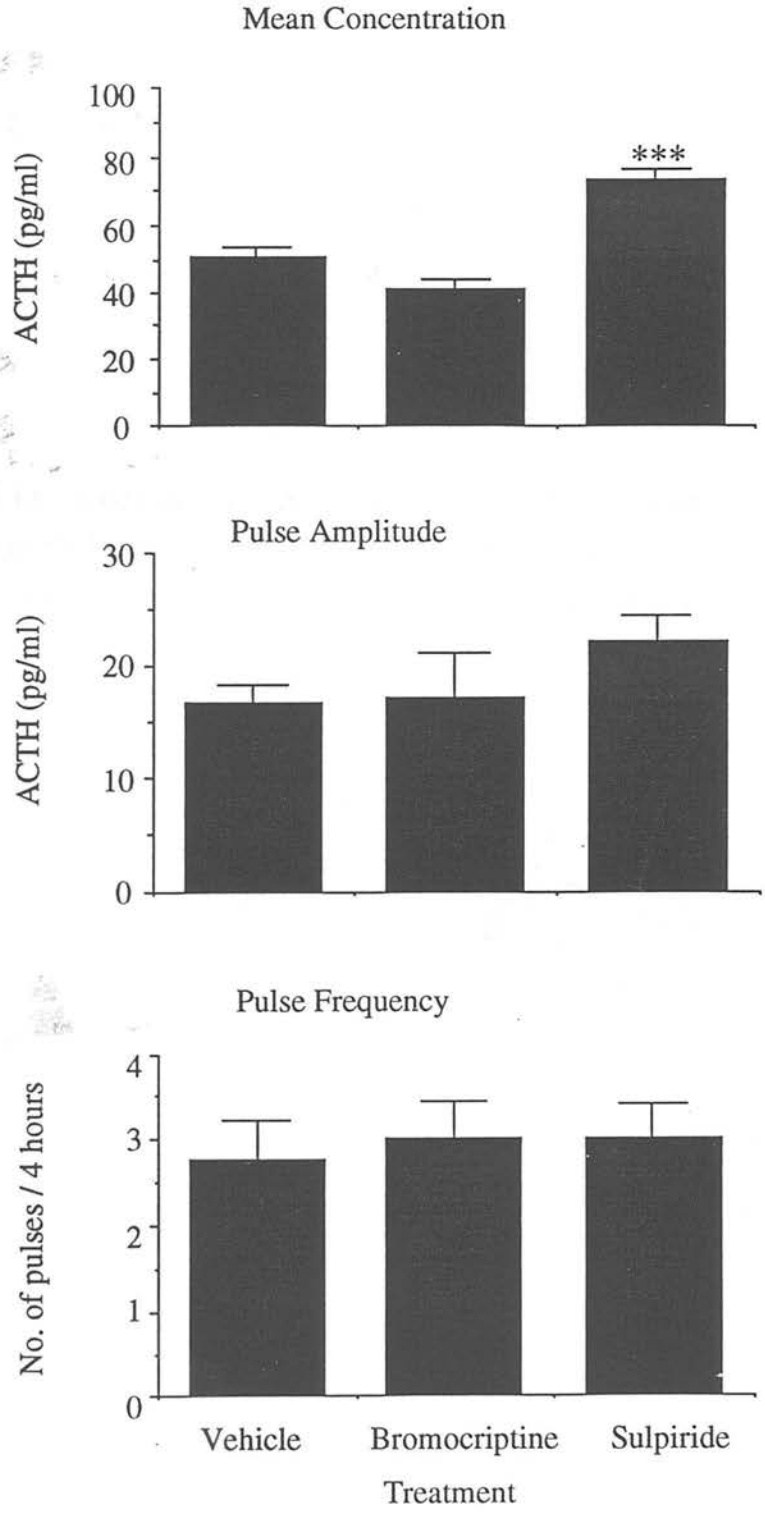
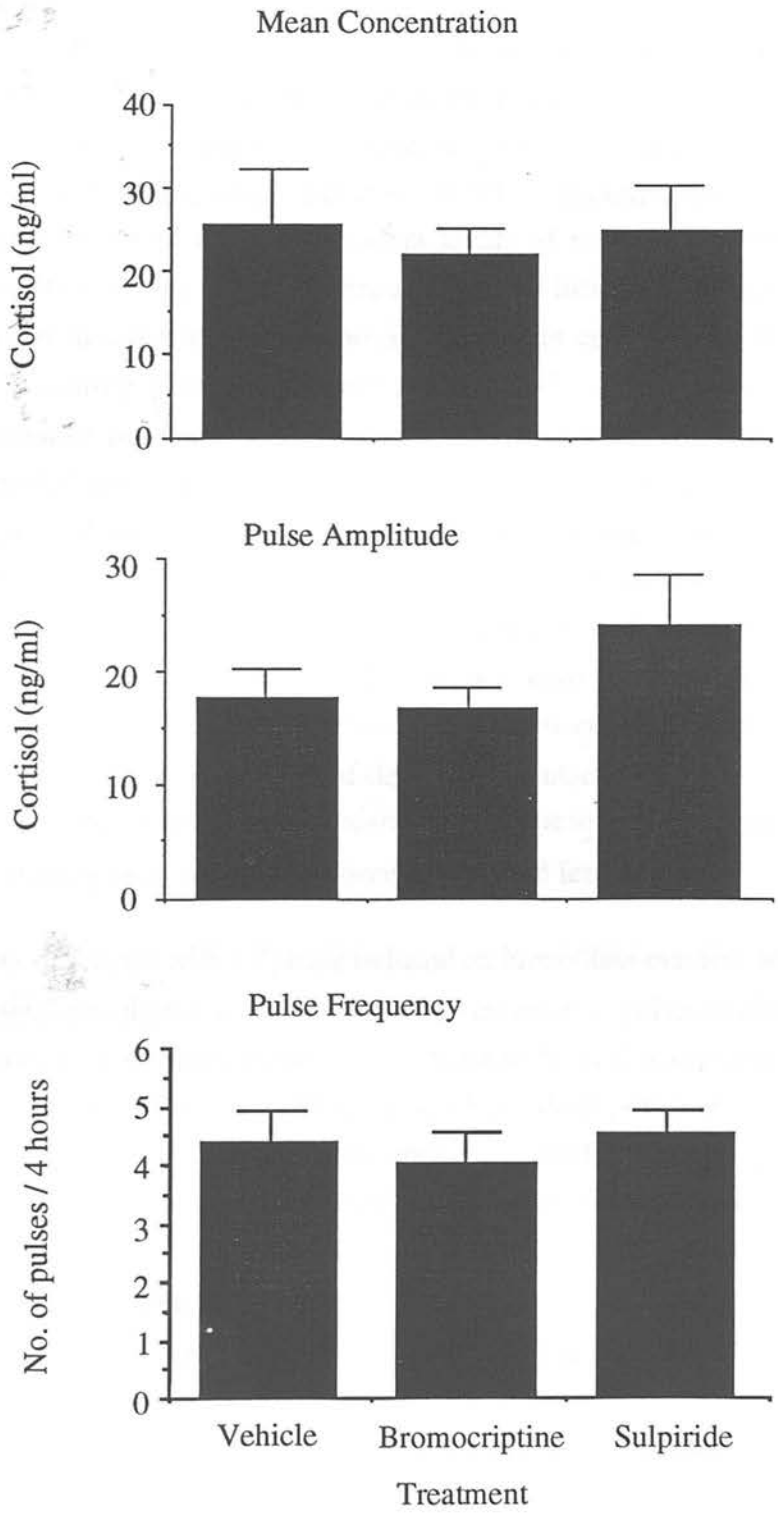

Mean Concentration
ACTH (pg/ml)
100
80
60
40
20
0
***
Pulse Amplitude
ACTH (pg/ml)
30
20
10
0
Pulse Frequency
No. of pulses / 4 hours
4
3
2
1
0
Vehicle
Bromocriptine
Sulpiride
Treatment

Figure 6.13. Analysis of cortisol pulse characteristics following 72 hour infusion of vehicle (n=8), bromocriptine (n=7) or sulpiride (n=12). There was no significant effect of any treatment of mean measured plasma cortisol concentration, pulse amplitude or pulse frequency.


Mean Concentration
Cortisol (ng/ml)
40
30
20
10
0
Pulse Amplitude
Cortisol (ng/ml)
30
20
10
0
Pulse Frequency
No. of pulses / 4 hours
6
5
4
3
2
1
0
Vehicle
Bromocriptine
Sulpiride
Treatment

pulsatile secretion of ACTH, α-MSH and β-endorphin into the adult circulation and presented a variety of profiles in which these hormones are secreted both in synchrony and asynchrony with each other. One such profile was the concomitant secretion of ACTH and α-MSH leading the authors to suggest that this represents secretion of both hormones from the anterior pituitary. The contribution of the anterior pituitary was assessed by chronically treating animals with dexamethasone. Together with decreasing plasma ACTH concentrations, dexamethasone significantly reduced the mean plasma levels of α-MSH and decreased α-MSH pulse amplitude whilst α-MSH interpulse interval increased. The authors suggested that α-MSH of anterior pituitary origin released in concert with ACTH contributes to the circulating plasma concentrations of α-MSH. However, the effects of dexamethasone in the study of Engler *et al.* (1989) could be accounted for by the recent identification of functional glucocorticoid receptors in the fetal intermediate lobe (Bertini, Westphal, deKloet and Kiss, 1989; Sheppard, Autelitano, Roberts and Blum, 1993). Engler *et al.* report that the decrease in plasma α-MSH concentrations following bromocriptine infusion is accompanied by a decrease in pulse frequency with no change in interpulse interval. This is at odds with the data presented in this chapter as we found that the bromocriptine-induced decrease in plasma α-MSH concentration reflects a significant decrease in pulse frequency such that pulsatile secretion is almost abolished. Indeed, in the present study α-MSH pulses were detected in only two of the 7 bromocriptine treated fetuses.

Treatment of fetuses with sulpiride induced an immediate massive release of α-MSH into the fetal circulation accompanied by an increase in pulse amplitude. These data demonstrate that α-MSH secretion is subject to tonic dopaminergic inhibition and suggests that α-MSH is stored in the fetal pituitary gland as a readily releasable pool. However, the fact that bromocriptine is capable of further inhibiting α-MSH concentrations suggests that α-MSH is not completely suppressed by these tonic inhibitory mechanisms. The more rapid response to the antagonist sulpiride when compared to the agonist bromocriptine is supportive of the concept of a releasable pool as infusion of the antagonist will act to release the pituitary gland from the inhibitory tone and allow rapid release of available α-MSH stores.

The results presented in this chapter are in accordance with those of Newman *et al.* (1987) who demonstrated stimulated plasma α-MSH secretion following a bolus

injection of the dopamine receptor antagonist metoclopramide to fetuses at day 116-138 gestation. The response to a bolus injection of metoclopramide is comparable to the response seen to sulpiride infusion in that there is a rapid increase in α-MSH secretion maximal 15 minutes after injection. The study of Newman *et al.* did not include any period of intensive sampling and therefore there is no indication of pulsatile secretion of α-MSH.

Following 72 hours of treatment, bromocriptine and sulpiride both significantly reduced α-MSH pulse frequency. One possible explanation for this apparent paradox may be that dopamine acts to regulate the level of basal secretion of α-MSH rather than influencing the generation of pulses. If this were the case, significantly elevated basal concentrations of α-MSH in response to sulpiride would act to mask pulses of smaller amplitude and so pulse frequency decreases. Similarly, bromocriptine treatment suppresses basal α-MSH concentrations to the extent that pulses are no longer evident, perhaps reflecting a decrease in pituitary α-MSH content. This suggests that something other than dopamine is acting to regulate the generation of α-MSH pulses. In this respect, CRH has been shown to stimulate the secretion of α-MSH from rat intermediate lobe cultures (Meunier, Lefevre, Dumont and Labrie, 1982), raising the possibility that CRH acts to regulate pulsatile secretion from the intermediate lobe, as it does in the anterior lobe of the pituitary gland.

The secretion of ACTH from the pituitary gland is unaffected by infusion of bromocriptine whilst 72 hour infusion of sulpiride significantly increases the plasma concentration of ACTH. Analysis of the pattern of ACTH secretion following the 72 hour infusion reveals that the increase in plasma ACTH concentration is independent of any effects on pulse amplitude or pulse frequency. Thus, dopaminergic regulation of ACTH secretion from the fetal pituitary gland reflects regulation of the basal secretion of ACTH. Bromocriptine treatment did not further suppress the level of ACTH in fetal plasma and suggests that the system is optimally suppressed, that the secretion of ACTH is largely outwith the inhibitory control of dopamine or that the dose of bromocriptine used was insufficient. Whilst the dose of bromocriptine was sufficient to induce a marked decrease in circulating α-MSH concentrations, it remains possible that the secretion of α-MSH is more responsive to bromocriptine than that of ACTH. The increased ACTH concentrations in response to sulpiride treatment may reflect an indirect action of dopamine on

anterior pituitary ACTH secretion via an action on hypothalamic CRH and/or AVP neurons. In this respect, dopaminergic innervation of the paraventricular nucleus has been demonstrated however, there is little data examining the effects of dopamine on CRH and/or AVP secretion. An alternative mechanism by which dopamine could influence ACTH secretion could be by direct action on the intermediate lobe. Dopaminergic regulation of the intermediate lobe is well established and it is interesting to speculate that the ACTH released in response to sulpiride-infusion is of intermediate lobe origin (see chapter 7). Indeed, this could account for the lack of effect on ACTH pulse characteristics following sulpiride infusion as the removal of dopaminergic inhibition may facilitate the release of a basal level of ACTH from the intermediate lobe whilst the normal pulsatile secretion of ACTH from the anterior pituitary remains unaffected.

Despite the increased plasma concentrations of ACTH and α-MSH following intravenous infusion of sulpiride, the concentration of cortisol in fetal plasma was unaffected. This was a surprising (and disappointing) result. It is possible that the fetal adrenal gland is unable to respond to increased ACTH in the fetal circulation at this stage of gestation. For this reason, the inclusion of an adrenal response challenge test at the end of the infusion period may have been helpful. However, in the absence of such a test, it remains unlikely that the adrenal gland was simply unresponsive during this stage of gestation as previous studies have demonstrated the presence of the cytochrome P450 steroid hydroxylase enzymes (Tangalatis *et al.*, 1990) necessary for cortisol biosynthesis at this stage of gestation. Moreover, these enzymes are ACTH-inducible and activate increased steroidogenesis in response to exogenous ACTH, further supporting the proposal that the ACTH released in response to sulpiride infusion is not acting at the fetal adrenal gland. The inability to stimulate the secretion of cortisol from the adrenal gland suggests that the ACTH released in response to sulpiride infusion is not bioactive. The IRMA system used in these studies is unable to distinguish between bioactive $ACTH_{(1-39)}$ and immunoreactive high molecular weight precursors. Therefore, manipulation of the dopamine system could influence the post-translational processing of POMC to favour production of immunoreactive fragment which are not involved in adrenal steroidogenesis. Alterations in the post-translational processing of intermediate lobe β-endorphin by dopamine has previously been demonstrated in the rat (Millington, O'Donohue and Mueller, 1987). Thus, it is possible that altered post-translational

processing of the POMC molecule in the intermediate lobe of the pituitary could result in the release of biologically inactive ACTH of intermediate-lobe origin.

In support of this hypothesis, surgical disconnection of the hypothalamus from the pituitary in the fetal sheep results in hypertrophy of the intermediate lobe and increased basal levels of ACTH (Antolovich *et al.*, 1991). However, in agreement with the data presented in this chapter, the increase in ACTH in response to HPD is not associated with an increase cortisol secretion, suggestive of the release of biologically inactive ACTH, which may originate in the intermediate lobe. The pulsatile secretion of other POMC-derived peptides has not been studied in the HPD fetal sheep. However, in the adult sheep this procedure is accompanied by the hypersecretion of ACTH, β-endorphin and α-MSH (Clarke, Clements, Cummins, Dench, Smith, Robinson and Funder, 1986).

The lack of an effect of elevated α-MSH on concentrations of cortisol in the fetal plasma contrasts considerably with the study of Llanos *et al.* (1979) who demonstrated that exogenous administration of α-MSH to fetal sheep between day 121-130 gestation stimulates cortisol concentrations. However, in this study a dose of 75μg α-MSH was administered which would be expected to elevate plasma α-MSH to concentrations of around 200ng/ml. This is far in excess of both the normal and sulpiride-induced concentrations found in the present study and suggests that their results are not physiologically relevant.

In conclusion, this study demonstrates the inherent ability of the fetal pituitary to secrete POMC-peptides in a pulsatile manner and illustrates the presence of a tonic dopaminergic inhibitory system regulating the release of ACTH and α-MSH from the pituitary gland during fetal life.

Chapter 7. Dopaminergic regulation of peptide content in the ovine fetal pituitary gland

7.1. Introduction

The studies presented in Chapter 6 illustrates the presence of a tonic dopaminergic inhibitory mechanism regulating the secretion of α-MSH and ACTH into the fetal circulation. As it is not always possible or practical to gain repeated access to the fetal circulation, previous studies have utilised determination of pituitary peptide content in an attempt to gain insight to the peptide stores present in the fetal pituitary. These studies are largely concerned with the ontogeny of ACTH-immunoreactive species in the pituitary during fetal life. Silman *et al.* (1979) used a variety of radioimmunoassays to characterise ACTH related peptides in fractions of fetal and adult pituitaries which had been extracted and subjected to Sephadex column chromatography. The authors identified a number of ACTH-related species of varying molecular weight. In addition to the presence of β-MSH, β-lipotrophin (β-LPH), γ-LPH, β-endorphin and ACTH, they reported the presence of three peaks of high-molecular weight which are believed to be precursors of ACTH. These high molecular weight immunoreactive species were predominant in the fetal pituitary whilst ACTH was the dominant peptide in the adult pituitary, suggesting differential processing of the POMC precursor molecule in the fetal and adult pituitary gland. These workers also reported that there was little or no evidence for the presence of α-MSH or CLIP in the pituitary gland of either fetal or adult sheep. A further study using isolated anterior and intermediate lobes of the pituitary revealed that the high molecular weight species were present only in the anterior lobe (Silman, Street, Holland, Chard, Falconer and Robinson, 1981).

More recently, and contrary to the suggestions of Silman *et al.* (1979; 1981), immunohistochemical identification of ir-ACTH has been demonstrated in the intermediate lobe of the fetal sheep pituitary gland (Mulvogue *et al.*, 1986; Matthews *et al.*, 1995; Chapter 5). In addition, intense immunostaining for α-MSH in the fetal intermediate lobe has been identified during the present series of experiments (Chapter 5). However, whilst immunohistochemical detection of peptides gives valuable information concerning the presence and cellular localisation of immunoreactive species it does not provide any information regarding the molecular nature of the peptides that are present. This is particularly important in

this instance as the peptides are derived from differential processing of the same precursor molecule.

The present study was designed to allow direct determination of pituitary ACTH and α-MSH content, and to examine what influence the dopaminergic regulation of POMC-derived peptide secretion described in Chapter 6 has on the pituitary content of these peptides. Specifically, fetal pituitaries removed following a 72 hour administration of either the dopamine agonist bromocriptine, the dopamine antagonist sulpiride or vehicle control to fetal sheep at day 131 gestation were dissected into anterior and intermediate lobes, subjected to peptide extraction and measurement of ACTH and α-MSH content by radioimmunoassay. In order to further characterise the nature of the immunoreactive forms of α-MSH and ACTH in the pituitary, extracts were separated on the basis of molecular weight using Sephadex chromatography prior to radioimmunoassay.

7.2. Materials and methods

7.2.1. Animals and surgery

11 sheep of mixed breed with known insemination dates were used in this study. Fetal catheterisation was carried out at day 125 - 126 gestation (term =145 days) as described previously in Chapter 3.

7.2.2. Experimental protocol

Beginning day 131 gestation, fetuses received an intravenous infusion of either the dopamine antagonist sulpiride (0.3mg/0.5ml/hr; n=4), the dopamine agonist bromocriptine (0.03mg/0.5ml/hr; n=4) or vehicle (0.1M tartaric acid in saline ; n=3) alone. Infusion continued for 72 hours. At various times during the 72 hour infusion period, a 0.2ml fetal blood sample was withdrawn for measurement of pO_2, pCO_2 and pH as described previously.

7.2.3. Tissue collection

At the end of the 72 hour infusion period, ewes were killed, fetuses removed and intact fetal pituitaries were collected immediately. The intermediate lobe of the pituitary gland was dissected free of the anterior lobe, weighed and snap frozen in liquid nitrogen. A small coronal slice, 2-3mm thick was removed from the middle

of the anterior lobe and placed in 4% paraformaldehyde, processed to paraffin wax and immunohistochemically stained for α-MSH as described in section 3.6. The remaining two pieces of anterior lobe were cut in the sagittal plane and opposing quarters were weighed and snap frozen together in liquid nitrogen. All frozen tissue samples were stored in liquid nitrogen prior to extraction.

7.2.4. Peptide extraction

Weighed tissue was added to 1ml of boiling 0.1M HCl/1M acetic acid (1:1 v/v) containing 250KIU/ml aprotinin and boiled for 5 minutes. The mixture was then cooled on ice and homogenised in a glass homogeniser (Uniform homogeniser; Jencons Scientific, Bedfordshire). The homogenate was removed to a fresh tube and the homogeniser rinsed with 500µl of the acid solution, which was added to the homogenate and 50µl was removed for determination of protein content. The homogenate was vortexed briefly and then the extracts were centrifuged at 10,000g (Beckman J2-21 Centrifuge; Beckman Instrument Inc., California). The supernatant was divided into aliquots which were freeze-dried and stored at -20°C.

7.2.5. Protein content

Protein concentrations in the tissue homogenates were determined using a commercially available assay kit (Biorad Protein Assay Dye, Biorad, California, USA). Tissue proteins were dissolved by boiling 50µl tissue homogenate in 950µl 0.1M NaOH for 30 mins. A protein standard curve was prepared by double diluting BSA (Sigma) in 0.1M NaOH in the range 25-1.56 µg/ml. 200µl of chromagen was added to 800µl of the sample or standard, the tubes were vortexed and incubated at room temperature for 30 minutes and the absorbance of the samples at 595nm (Uvikon 880 Spectrophotometer, Zurich, Switzerland) was measured. All samples and standards were assayed in triplicate. Samples were assayed at a range of dilutions to ensure they read on the standard scale. The protein concentrations in the samples were calculated relative to the readings for the standard curve.

7.2.6. Peptide content

Lyophilised tissue extracts were reconstituted in the appropriate assay buffer and assayed for αMSH and ACTH content by radioimmunoassay (section 3.4) and also for ACTH using the IRMA method (section 3.5). In order to ensure that peptide

concentrations were assayed on the most sensitive part of the standard curve, a range of dilutions were assayed for each sample. The content of hormone in the tissues was then expressed relative to wet weight of the tissue.

7.2.7. Column chromatography

Sephadex G50 superfine (Sigma) was swollen for 2 hours at room temperature in 0.1M HCl. The slurry was then packed into a 50cm x1cm glass column (Amicon Ltd.) under gravity. The column was then transferred to a cold room (4°C) and perfused overnight with 0.1M HCl at a rate of 4ml/hour (Watson Marlowe Pump, Type 202U; Falmouth, Cornwall). The column was primed with 5ml 10% BSA in water to prevent binding to non-specific sites in the gel matrix. The column was calibrated by addition of a 'cocktail' containing α-MSH and ACTH or CLIP in 1ml HCl which was eluted at a flow rate of 4ml/hour. 800µl fractions were collected by an electronic fraction collector (Gilson Model 203). Fractions were divided into two aliquots, frozen, lyophilised and stored at -20°C. Blue dextran (2mg/ml, Pharmacia) exclusion was used to determine the void volume of the column. Selected pituitary extracts (two extracts from each treatment group) were diluted in 0.1M HCL and applied to the column and fractions collected, frozen and lyophilised as described above. Column fractions were assayed for α-MSH and ACTH by radioimmunoassay as described in section 3.4.

7.2.8. Data analysis

Intermediate lobe weights, protein content and α-MSH and ACTH levels in intermediate and anterior lobes of the pituitary from the three treatment groups were compared by ANOVA followed by post-hoc t- test. All data are expressed as mean ± standard error of mean (SEM) and $p<0.05$ was considered to be significant.

7.3. Results

7.3.1. Efficacy of dissection

Tissue slices removed from the anterior pituitary were sectioned and stained immunohistochemically for α-MSH, as a marker of intermediate lobe melanocyte presence to determine the efficiency of separation of the intermediate lobe from the anterior lobe of the pituitary. Figure 7.1. shows representative sections of anterior

pituitary stained for α-MSH following intermediate lobe dissection. The lack of immunostaining for α-MSH suggests that the intermediate lobe was removed in its entirety at the time of dissection and that anterior pituitary tissues collected are not contaminated by the presence of intermediate lobe tissue. A section of fetal pituitary with an intact intermediate lobe was included as a positive control.

7.3.2. Fetal pituitary content of ACTH and α-MSH

The average wet weight of the intermediate lobe from vehicle-treated control fetuses was 17.13 ± 0.51mg. The mean intermediate lobe weight from pituitaries in each treatment group are shown in Table 7.1. The average intermediate lobe weight was unaffected by treatment with either bromocriptine or sulpiride when compared to vehicle treated controls (Table 7.1).

Treatment	**Mean intermediate lobe weight (mg ± SEM)**
vehicle	17.13 ± 0.51 (n=3)
bromocriptine	14.22 ± 1.45 (n=4)
sulpiride	18.85 ± 2.53 (n=4)

Table 7.1. Intermediate lobe weight for all three treatment groups. Data expressed represent the mean values for all tissues collected in each treatment group ± SEM.

Pituitary α-MSH content expressed relative to wet tissue weight is shown in Figure 7.2. α-MSH was present in both the intermediate and anterior lobes of the pituitary. The intermediate lobe of the pituitary contained large amounts of α-MSH and the content was unaffected by treatment with either bromocriptine or sulpiride. Significantly less α-MSH was identified in the anterior pituitary and this too was not significantly altered by infusion of either bromocriptine or sulpiride. Pituitary ACTH content was measured by both radioimmunoassay, which identifies many ACTH-like peptides ($ACTH_{(1-39)}$, $ACTH_{(1-10)}$, $ACTH_{(1-24)}$ and α-MSH all show significant cross-reactivity) and the more specific two-site immunoradiometric assay which recognises specifically the $ACTH_{(1-39)}$ molecule. The ACTH RIA

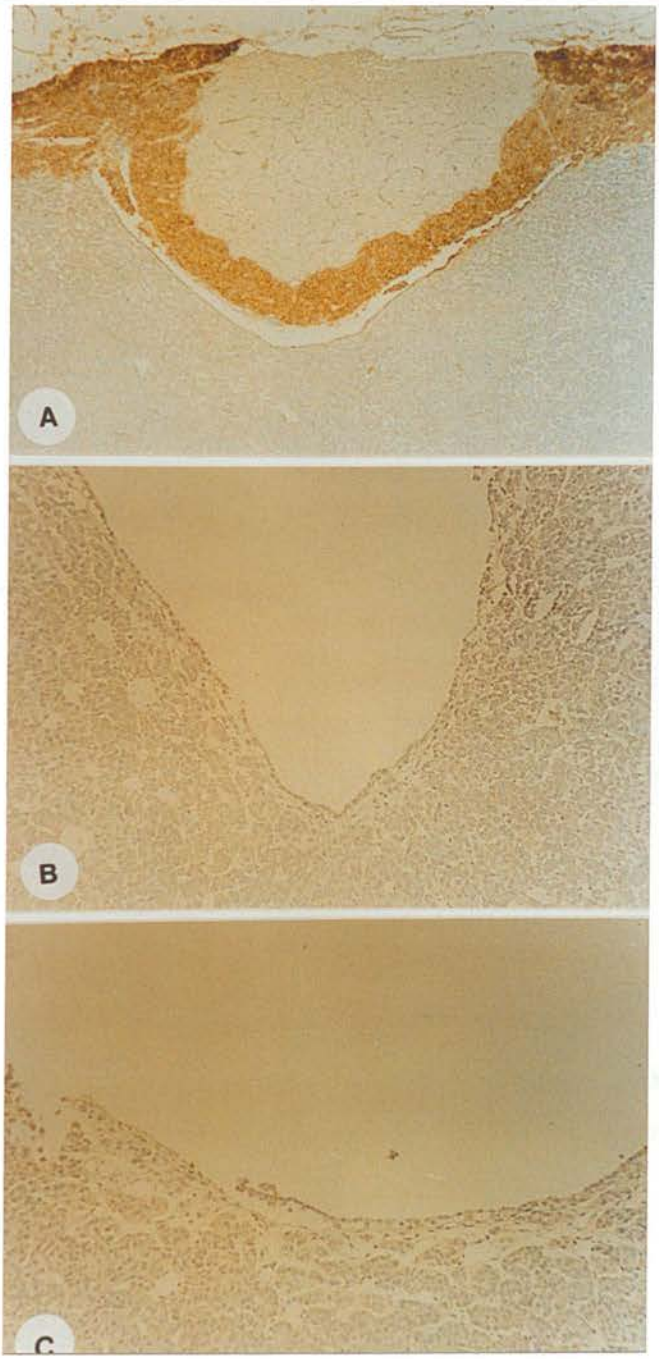


Figure 7.1. Immunohistochemical staining for α-MSH in anterior pituitary sections following intermediate lobe dissection. 7.1(a) illustrates positive immunostaining for α-MSH in a section of intact fetal pituitary. Representative pituitary sections following intermediate lobe dissection are shown in Figure 7.1(b and c).

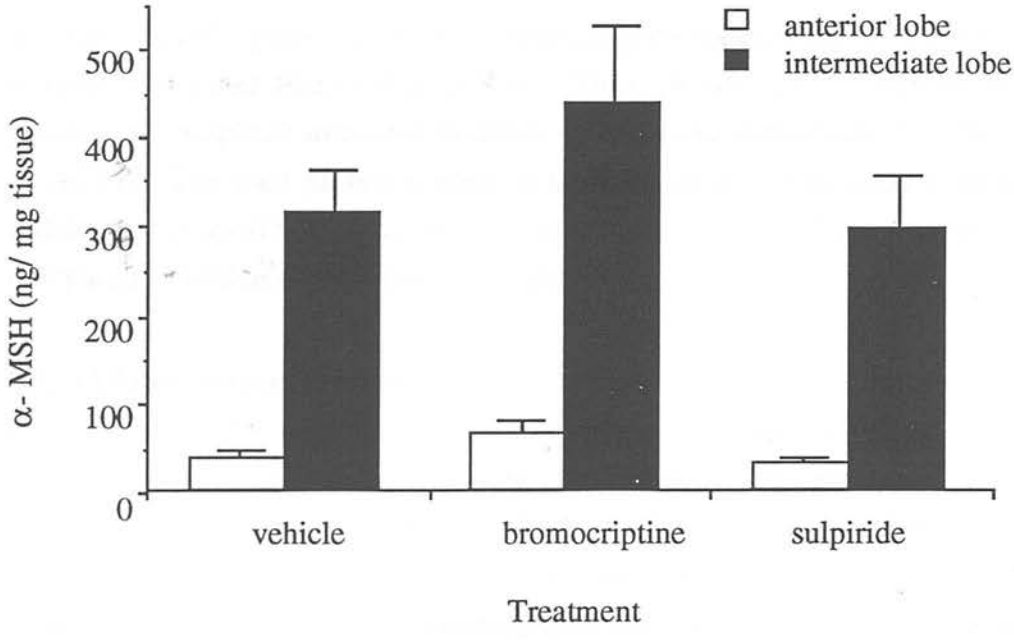


Figure 7.2. Content of α-MSH in the anterior (open bars) and intermediate (closed bars) lobes of the pituitary. Peptide contents were expressed as ng/mg tissue. Data shown are the mean ± SEM of all tissues from each treatment group.

consistently measured more ACTH-immunoreactive species than ACTH IRMA (Figure 7.3). In both systems, ACTH was most abundant in the anterior pituitary and the content was unaffected by treatment with either bromocriptine or sulpiride. ACTH was also measured in the intermediate lobe both by RIA and IRMA. Whilst ACTH content in treated groups did not differ significantly from vehicle -treated controls, the intermediate lobe ACTH content, as measured both by RIA and IRMA was significantly greater ($p<0.05$) in bromocriptine-treated fetuses when compared to sulpiride-treated fetuses (Figure 7.3b). Thus, bromocriptine treatment tended to increase and sulpiride treatment tended to decrease the intermediate pituitary content of ACTH. The total protein content of the anterior and intermediate lobes of the pituitary was unaffected by infusion with either bromocriptine or sulpiride when compared to vehicle-treated controls (Figure 7.4).

3.3. Column chromatography

Following column calibration, pituitary extracts from vehicle, bromocriptine and sulpiride infused fetuses were subjected to column chromatography. Figure 7.5 shows immunoreactive α-MSH and ACTH in fractions obtained from a representative anterior pituitary extract from each treatment group following Sephadex chromatography. Chromatography was performed on two pituitaries from each treatment group and both gave similar results. Two immunoreactive peaks were detected in anterior pituitary extracts from all three treatment groups. One peak eluted in fractions 26-31 and co-eluted with synthetic α-MSH. The second immunoreactive peak cross-reacted in the ACTH RIA and eluted in fractions 20-25, corresponding to elution of synthetic $ACTH_{(1-39)}$. An additional peak of immunoreactivity which cross-reacted in the ACTH RIA was identified in fractions 10-15 of vehicle and bromocriptine treated anterior pituitary extracts and in fractions 14-18 of anterior pituitary extracts from sulpiride-treated fetuses. The intermediate pituitary also contained a large peak of immunoreactive α-MSH which eluted in the same position as synthetic α-MSH and two peaks which contained ir- ACTH (Figure 7.6). As with the anterior pituitary extracts, ir-ACTH was identified in a peak that corresponded with the elution of synthetic $ACTH_{(1-39)}$ (fractions 20-25) and also a peak of higher molecular weight. Immunoreactive ACTH was also identified in fractions corresponding to the elution of synthetic $ACTH_{(18-39)}$ (CLIP). The profile of α-MSH and ACTH immunoreactive species resulting from intermediate pituitary extracts was unaffected by treatment with bromocriptine or sulpiride.

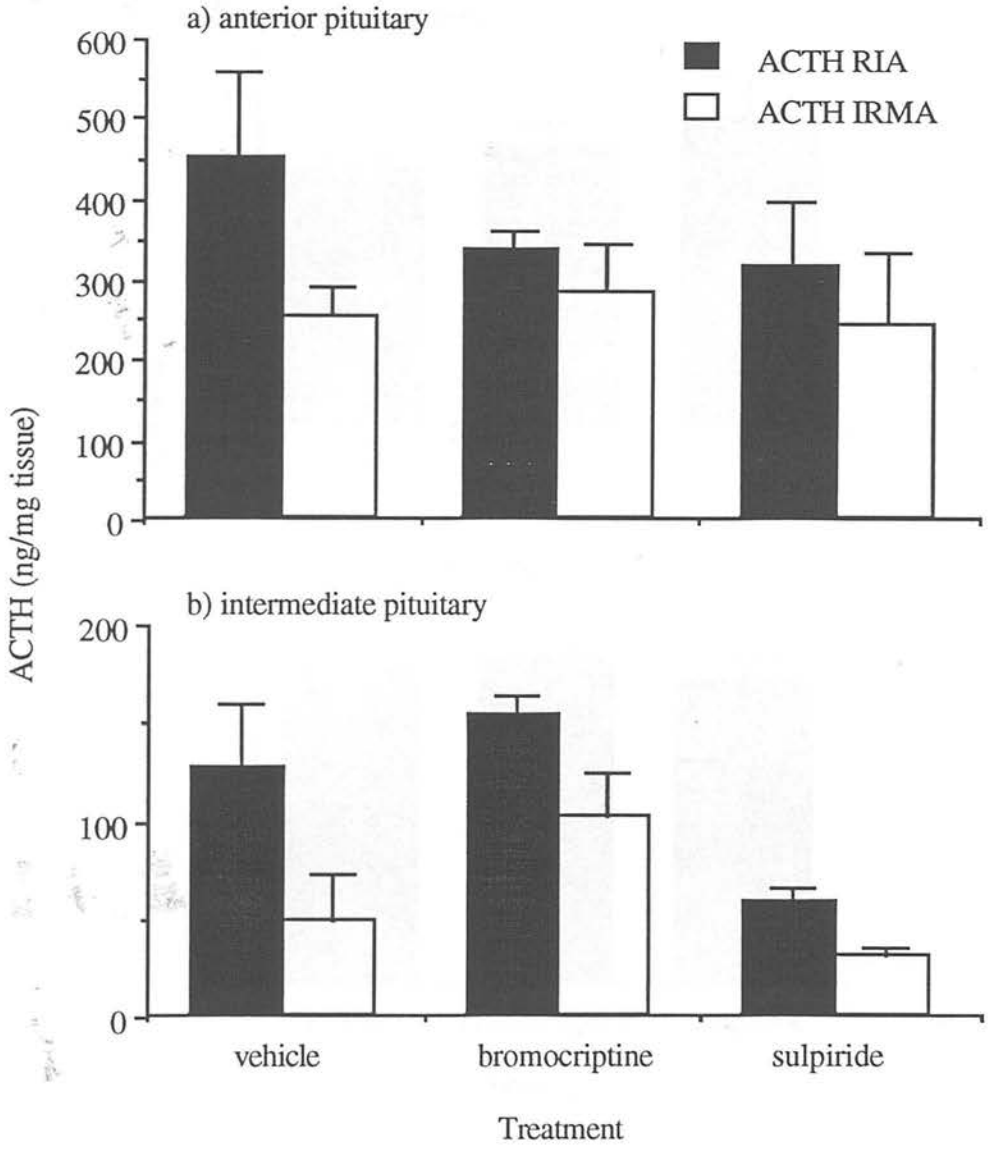


Figure 7.3. Content of ACTH in the (a) anterior and (b) intermediate lobes of the pituitary, measured by RIA (open bars) and IRMA (closed bars). Peptide contents were expressed as ng/mg tissue. Data shown are the means ± SEM of all tissues from each treatment group.

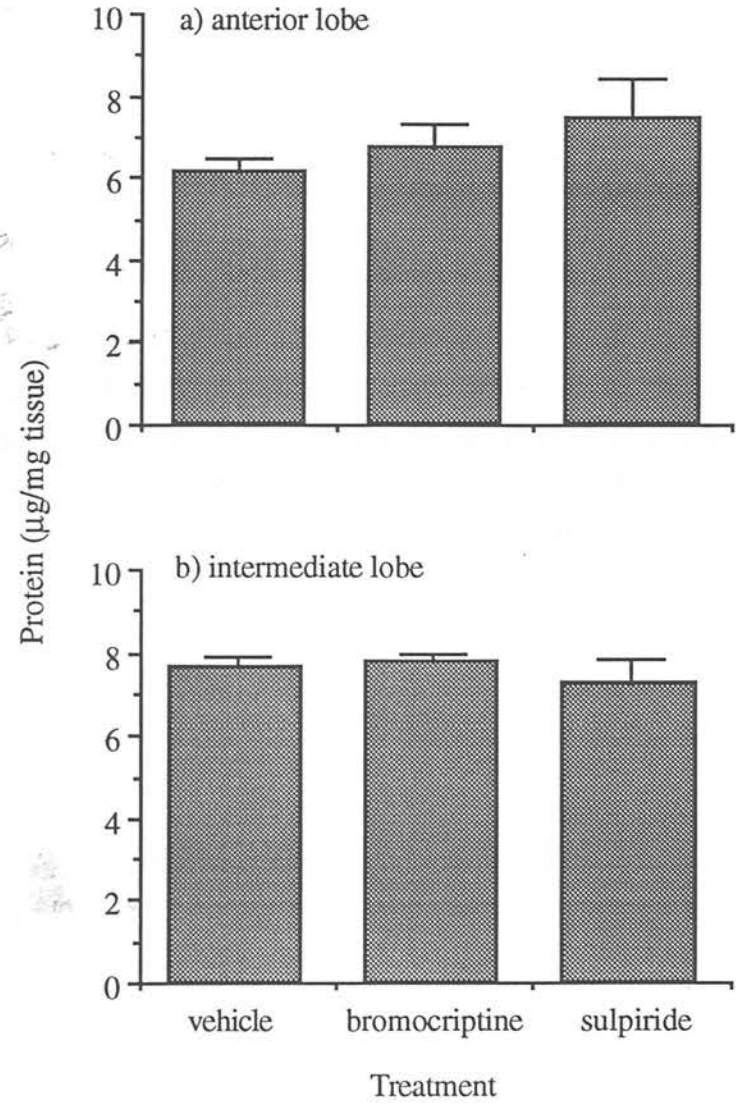


Figure 7.4. Total protein content in extracts from a) anterior lobe and b) intermediate lobes of vehicle, bromocriptine and sulpiride treated pituitaries. Protein contents are expressed as µg protein/mg tissue. Data shown are the mean ± SEM of all tissues from each treatment group.

Figure 7.5. ACTH and α-MSH immunoreactivity determined after chromatography of anterior pituitary extracts prepared from a) vehicle treated, b) bromocriptine treated and c) sulpiride treated fetuses. The elution positions of standard ACTH, α-MSH and CLIP are shown by the filled bars. Blue dextran (M.Wt 2 X 10^6) eluted at Vo.

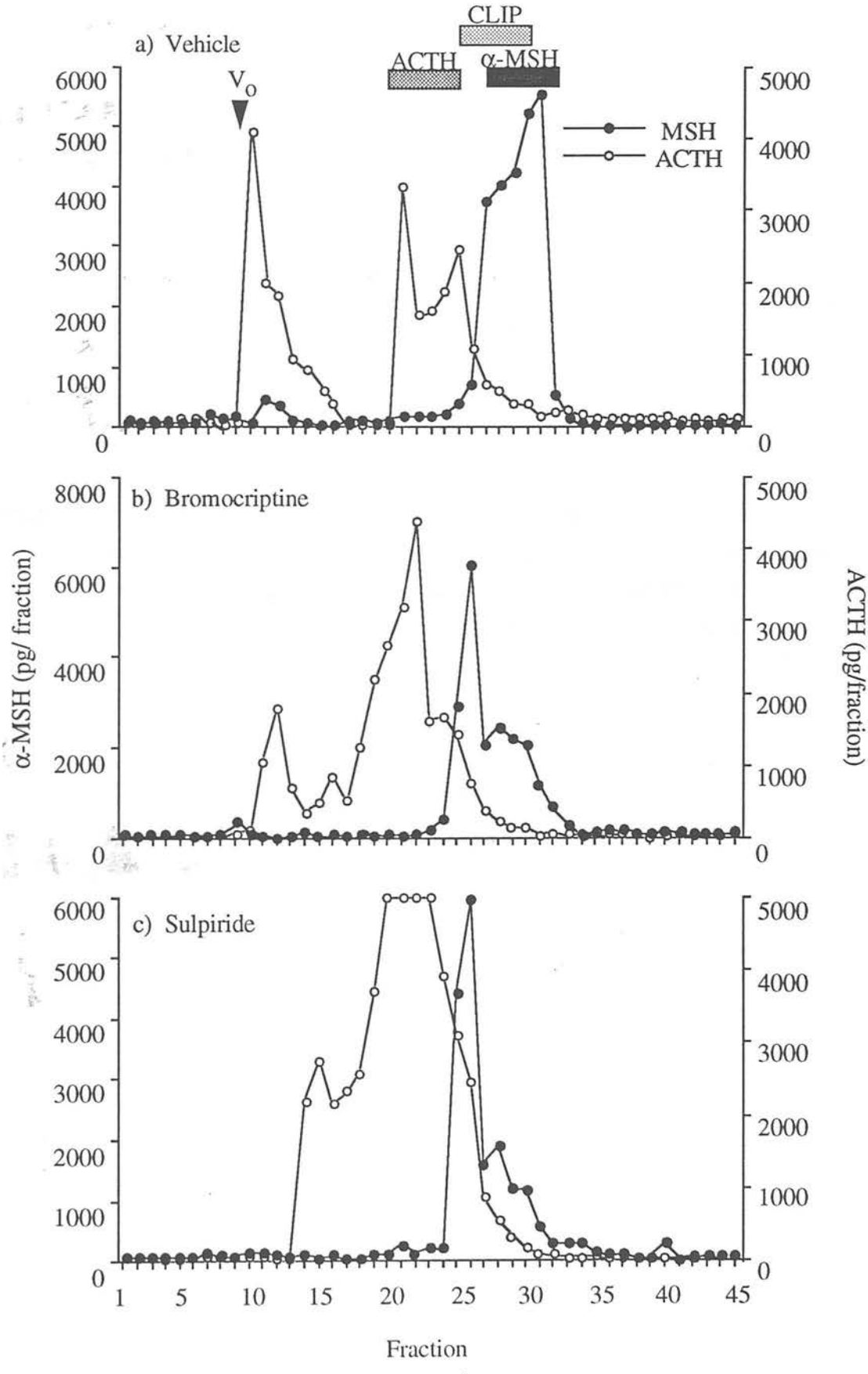
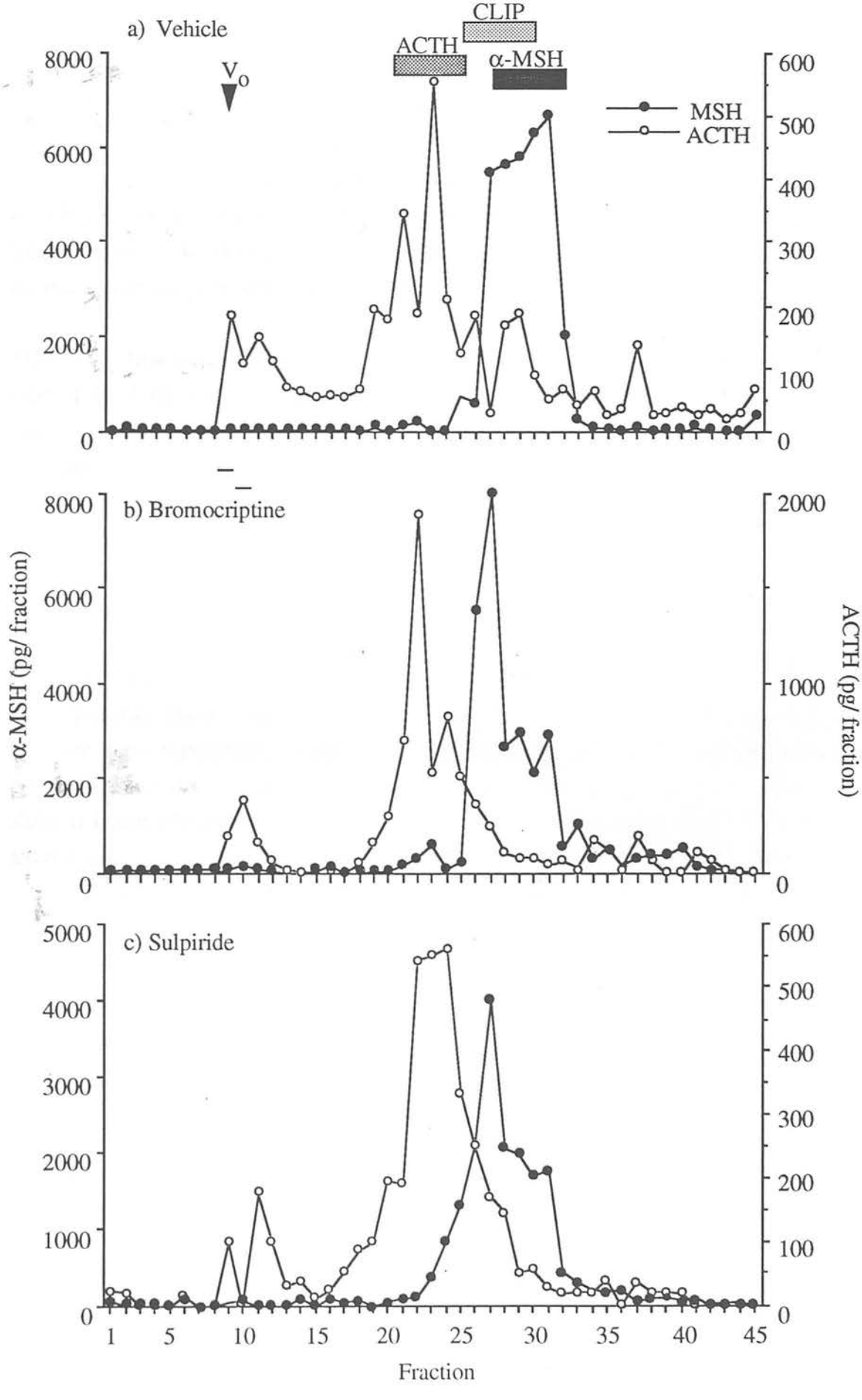

a) Vehicle
CLIP
ACTH
α-MSH
V_O
MSH
ACTH
b) Bromocriptine
c) Sulpiride
α-MSH (pg/ fraction)
ACTH (pg/fraction)
Fraction

Figure 7.6. ACTH and α-MSH immunoreactivity determined after chromatography of intermediate lobe extracts prepared from a) vehicle treated, b) bromocriptine treated and c) sulpiride treated fetuses. The elution positions of standard ACTH, α-MSH and CLIP are shown by the filled bars. Blue dextran (M.Wt 2 X 10^6) eluted at Vo.


a) Vehicle
CLIP
ACTH
α-MSH
V_o
MSH
ACTH
b) Bromocriptine
c) Sulpiride
α-MSH (pg/ fraction)
ACTH (pg/ fraction)
Fraction
0
2000
4000
6000
8000
1000
3000
5000
100
200
300
400
500
600
1000
2000
1
5
10
15
20
25
30
35
40
45

7.4. Discussion

The aim of this study was to investigate the effects of intrafetal infusion of bromocriptine and sulpiride on the pituitary content of ACTH and α-MSH. ACTH and α-MSH were identified in both the anterior and intermediate lobes of the pituitary. The content of α-MSH and ACTH in the anterior and intermediate lobes of the pituitary was not significantly altered by treatment with either bromocriptine or sulpiride when compared to vehicle-treated controls. However, the intermediate lobe content of ACTH tended to increase following bromocriptine treatment and decrease in response to sulpiride treatment.

This is the first demonstration of the presence of $ACTH_{(1-39)}$ in the intermediate lobe of the fetal pituitary measured using a specific two-site immunoradiometric assay. The IRMA system used in this study recognises the $ACTH_{(1-39)}$ molecule but does not recognise either α-MSH or CLIP which are characteristically produced in the intermediate lobe by processing of ACTH. However, from the present study it is not possible to ascertain whether this ACTH is bioactive as well as immunoreactive. Further studies investigating the effects of pituitary extracts on cortisol secretion would be needed to clarify this matter.

When investigating peptide content it is important to remember the dynamic nature of the pituitary gland. Thus, the amount of peptide present in the pituitary gland at any one time represents a balance between the rate of peptide synthesis and processing and that of peptide secretion. From investigation of peptide content alone it is not possible to determine whether any change in content represents an altered rate of peptide synthesis or an altered rate of peptide secretion. In addition, as determination of peptide content represents a static measurement it may not be fully representative of the functional response of the pituitary unless it is examined in concert with measures of peptide secretion and synthesis. For this reason, the lack of effect of bromocriptine and sulpiride on pituitary α-MSH and ACTH content in the present study does not necessarily imply a lack of response on the part of the pituitary gland.

Hypothalamo-pituitary disconnection in the adult sheep results in a two-fold increase in intermediate pituitary α-N-acetylated endorphin (NacEP) and α-MSH content (Smith, Wallace, Clarke and Funder, 1989). In order to determine whether

this response reflected the loss of dopaminergic input to the intermediate lobe, adult sheep were treated with either bromocriptine or haloperidol for 10 days. In consonance with the results of the present study, the intermediate lobe α-MSH content was increased by haloperidol and decreased by bromocriptine although these effects failed to reach significance (Smith *et al.*, 1989). By contrast, the intermediate lobe content of ir-NacEP was significantly altered by treatment with both bromocriptine and haloperidol.

The influence of hypothalamic dopaminergic input on pituitary α-MSH content has previously been studied in adult rats bearing electrothermic lesions of the mediobasal hypothalamus (Tilders and Smelik, 1978). Eight hours after lesioning pituitary α-MSH content was decreased by 75% when compared to non-lesioned controls, and this fall could be prevented by bromocriptine treatment. In agreement with the data presented in this chapter, the authors reported no effect of bromocriptine on pituitary α-MSH content of intact control rats. A single injection of the dopamine antagonist haloperidol induced a significant reduction in pituitary α-MSH content which was maximal at 4-5 hours post-injection. However, despite continued elevation of plasma α-MSH concentrations in response to sulpiride infusion (Chapter 6) there was no significant change in pituitary α-MSH content in the present study. One possible explanation for the lack of effect in the present work is the reduced daily dose of antagonist administered to the fetuses. In the present study fetuses received a total of 21.6 mg sulpiride over the 72 hour period. The average fetal weight at post-mortem was 4.2kg equating to a dose of around 5mg/kg over the 72 hour period. This is comparable with the range of doses used by Tilders and Smelik (1978) who administered a variety of doses and found significant effects at 2.5 and 5.0 mg/kg. However, the 5.0mg/kg dose used by Tilders and Smelik was administered as a single injection whereas in the present study the continuous mode of administration resulted in fetuses receiving only 1.7mg/kg/day. It is possible that the continuous infusion used in the present study was insufficient to induce lasting changes at the level of pituitary content. Indeed, Tilders and Smelik were unable to influence pituitary α-MSH content at doses of 1.0 and 0.6 mg/kg. In addition, the present study and that of Tilders and Smelik (, 1978) utilised different antagonists and as haloperidol is a more potent antagonist than sulpiride, displaying greater affinity for anterior pituitary dopamine binding sites (Enjalbert and Bockaert, 1982), direct comparison between doses may not be appropriate.

Conversely, it may be that the dose of sulpiride administered during the present study was sufficient to influence pituitary content but that the chronic nature of the infusion allows the pituitary to compensate and adjust peptide contents accordingly. Thus, the rapid increase in circulating α-MSH concentrations in response to sulpiride shown in Chapter 6 may indeed result in a transient decrease in pituitary content but, by the end of the 72 hour infusion period the α-MSH content is restored to control levels. One possible mechanism by which the pituitary can readjust the intermediate lobe α-MSH content in the face of continuous hypersecretion is by alteration of the rate of α-MSH biosynthesis. This hypothesis is supported by the demonstration that reduced α-MSH content in response to haloperidol is accompanied by an increased capacity of intermediate lobe cells to synthesis α-MSH (Beaulieu *et al.*, 1984) and also an increased level of POMC mRNA. As mentioned previously, the maintenance of intermediate lobe content in the present study may also be reflected in the level of POMC gene expression and this possibility is investigated further in Chapter 8.

In contrast to the study of Tilders and Smelik (1978) and in support of the hypothesis of altered biosynthesis, a biphasic response of pituitary α-MSH content following bromocriptine treatment has been reported (Beaulieu *et al.*, 1984). Adult rats treated with bromocriptine for 2 days displayed a significant increase in intermediate lobe content of α-MSH. This increase in α-MSH content was coupled with a decrease in the synthesis of α-MSH like peptides. However, after 5 days of treatment with bromocriptine there was a marked reduction in intermediate lobe α-MSH content and following long term administration for up to 21 days the α-MSH content of the intermediate lobe was decreased by 70% when compared to controls. This is at odds with the present study in which 3 day infusion of bromocriptine did not significantly alter the pituitary content of α-MSH. However, it is possible that the 3 day period in the present study represents the transition period in the biphasic response to bromocriptine reported by Beaulieu and co-workers. In the study of Beaulieu *et al.*, rats were treated with bromocriptine administered as a daily injection at a dose of 10mg/kg/day which is significantly greater than the doses used both in the present study and in that of Tilders and Smelik (1978). In addition, the minimum dose of 0.3mg/kg/day for 4 days tested by Beaulieu et al.,which resulted in reduced levels of POMC synthesis, was almost 2 times greater than the dose used in the present study and suggests that the dose and mode of administration, together

with the time-frame of the response may be critically important in studies such as this.

Beaulieu *et al.* (1984) also reported a 39% decrease in total protein content in the intermediate lobes of bromocriptine treated rats when compared to vehicle-treated controls. No such change was identified in the present study. However, this is perhaps not surprising as there was no corresponding decrease in intermediate lobe content of peptide in response to bromocriptine.

In the present study, there was no significant effect of sulpiride on intermediate lobe ACTH content when compared to vehicle-treated controls. However, ACTH content of the intermediate lobe tended to decrease in response to sulpiride infusion and increase following bromocriptine infusion. It is conceivable that, given increased group sizes or perhaps a longer infusion period, intermediate lobe ACTH content may be significantly altered. Reduced intermediate lobe ACTH content in response to sulpiride infusion would be consistent with the hypothesis that sulpiride acts to increase the rate of secretion of ACTH from the intermediate lobe (see Chapter 6 discussion). However, if this was the case it is difficult to explain the lack of effect on α-MSH content when circulating levels of α-MSH are also significantly elevated by sulpiride infusion. Indeed, if the hypothesis of altered biosynthesis to compliment an altered rate of secretion was correct then it is not unreasonable to expect that pituitary ACTH and α-MSH content be regulated in the same manner. However, it is possible that the reduced content of ACTH in the intermediate lobe reflects an increased rate of processing of ACTH to produce α-MSH. Thus, ACTH is more rapidly processed to α-MSH resulting in maintained pituitary content of α-MSH coupled with a decreased pituitary content of ACTH. Support for this hypothesis comes from the finding that expression of the prohormone convertase enzymes PC1 and PC2 is regulated by dopamine (Day *et al.*, 1992). Levels of PC1 and PC2 mRNA in the intermediate lobe of adult rats significantly increased following haloperidol treatment and decreased following treatment with bromocriptine (Day *et al.*, 1992). Corresponding changes in POMC mRNA levels have also been reported (Day *et al.*, 1992). Interestingly, intermediate lobe PC2 mRNA levels were increased to a greater extent than PC1 and POMC mRNA levels for an identical dose of haloperidol (4-6 fold compared with 2-3 fold respectively). This differential regulation of PC1 and PC2 gene expression provides a mechanism for increased post-translational processing of the POMC precursor whereby ACTH

produced in the intermediate lobe following enzymatic cleavage of the precursor molecule by PC1 would be rapidly processed to α-MSH by the action of PC2.

The further possibility exists that regulation of peptide content in the fetal pituitary in response to altered rates of secretion occurs by mechanisms which are distinct from those utilised in the adult. However, the very similar results of Smith *et al.* (1989) suggest that this is not the case. In addition, all the evidence collated so far suggests that dopaminergic regulation of the fetal pituitary utilises many of the same mechanisms in the fetus as in the adult with respect to peptide secretion and gene regulation and therefore it seems at least possible that similar regulation of peptide content will occur. It remains possible, however, that regulation of pituitary function in the sheep is distinct from that in the rat. If this were the case then extrapolation from the studies of adult rats may be misleading.

In agreement with the results presented in this chapter, Smith *et al.* (1989) reported that the elution profiles of α-MSH immunoreactivity from bromocriptine and haloperidol treated sheep intermediate lobe extracts, subjected to HPLC analysis did not differ from that of vehicle-treated controls. In contrast, the pattern of NacEP processing observed in the inermediate lobe extracts of haloperidol treated sheep was similar to that obtained from HPD sheep, suggestive of dopaminergic regulation of post-translational processing in the intermediate lobe of the adult sheep.

Using Sephadex G100 chromatography, Silman *et al.* (1979) identified three peaks of high molecular weight which were distinct from β-MSH, β-LPH, γ-LPH, β-endorphin and ACTH. The 3 high molecular weight species were present in relatively greater proportions in the fetus than in the adult sheep pituitary demonstrating the existence of differential processing for the POMC molecule in the fetal pituitary compared to the adult pituitary gland. Dissection of the fetal pituitary and investigation of the anterior and intermediate lobes of the pituitary separately revealed that 2 of the high molecular weight peaks were present in the intermediate pituitary in significantly reduced quantities (Silman *et al.*, 1981). This concurs with the result of the present study in which a high molecular weight peak of ACTH-immunoreactivity was evident in the anterior lobe and, in reduced amounts, in the intermediate lobe of the pituitary suggestive of similar processing mechanisms in the anterior and intermediate lobes of the pituitary. However, Silman and co-workers failed to detect a peak corresponding to $ACTH_{(1-39)}$ in intermediate lobe extracts.

This differs significantly from the findings in the present study as intermediate lobe extracts contained significant amounts of $ACTH_{(1-39)}$ immunoreactivity as detected both by IRMA and by RIA following Sephadex chromatography. The marked difference in these results is difficult to explain. The possibility of anterior pituitary contamination of intermediate lobe extracts in the present study cannot be ruled out. However, this seems unlikely as great care was taken during the dissection procedure and subsequent histological examination of the coronal pituitary slice confirmed that the intermediate lobe had been cleanly removed. It is also feasible that the $ACTH_{(1-39)}$ detected in the present study arises from degradation of ACTH precursors during the extraction process and a protease inhibitor was included in the extraction procedure to reduce this possibility. Furthermore, the presence of substantial immunoreactivity for ACTH in the fetal intermediate lobe at this stage (see Chapters 5 and 8) suggests that the intermediate lobe does contain significant amounts of ACTH at this time in development. In addition, Silman *et al.* (1981) reported that the there was little evidence for the existence of α-MSH or CLIP in the pituitary gland of the fetus. In the present study however, α-MSH was evident in the intermediate and anterior lobes of the pituitary as detected both by direct radioimmunoassay of pituitary extracts and following Sephadex chromatography. This too conforms with the presence of abundant α-MSH immunostaining in the intermediate lobe (Chapters 5 and 8). In agreement with the present study, Smith and Funder (1988) reported that the anterior and intermediate lobes of the sheep pituitary contained significant α-MSH immunoreactivity characterised by HPLC analysis.

The presence of high molecular weight species of ACTH in the fetal pituitary gland has been demonstrated by others (Brieu and Durand, 1989a) and these molecules have been shown to antagonise ACTH-induced cortisol secretion from adrenal cells in vitro (Jones and Roebuck, 1980). As the relative proportion of high molecular weight forms of ACTH to $ACTH_{(1-39)}$ falls with advancing gestation (Roebuck *et al.*, 1980) it is possible that the changing ratio of high molecular weight ACTH to ACTH(1-39) plays a role in the late gestation cortisol surge from the fetal adrenal gland. The results of the present study would suggest that the intermediate lobe may contribute to the pool of high molecular weight ACTH precursors and could be an important source of $ACTH_{(1-39)}$ during fetal life. There was no evidence in the present study for dopaminergic modulation of POMC processing in the fetal

intermediate lobe as the profiles of immunoreactive ACTH and α-MSH species were unaltered by treatment with bromocriptine or sulpiride. However, in anterior pituitary extracts from sulpiride treated fetuses there was a tendency for the high molecular weight ACTH-immunoreactive peak to shift to the right, indicative of a lower molecular weight. It is possible that this peak represents the 'intermediate' ACTH described by Brieu and Durand (1989a) which eluted between the void volume of the column and standard $ACTH_{(1-39)}$. In that study, large amounts of 'big' ACTH (eluting in the void volume) and 'intermediate' ACTH were identified in extracts of cultured fetal pituitary cells and in total pituitary extracts, whereas the predominant form of ACTH immunoreactivity released into the incubation medium was $ACTH_{(1-39)}$. However, some 'intermediate' ACTH was released into the incubation media and this was found to be highly steroidogenic. By comparison, 'big' ACTH had only very weak steroidogenic activity. Thus, it is possible that the smaller molecular weight ACTH-immunoreactive molecule induced by sulpiride treatment in the present study represents a change in processing of the POMC molecule in the anterior pituitary to favour the production of a bioactive ACTH molecule. An in vitro bioassay of column fractions is needed to further elucidate the nature of the ACTH-immunoreactive peaks in the present study.

In conclusion, the present study demonstrates the presence of ACTH in both the anterior and intermediate lobes of the fetal pituitary. Immunoreactive ACTH was evident in both lobes as $ACTH_{(1-39)}$ and as a higher molecular weight species. The intermediate pituitary ACTH content was regulated by sulpiride and bromocriptine treatment. In addition, α-MSH was evident in both the anterior and intermediate lobes and pituitary content was unaltered by treatment with bromocriptine or sulpiride. These data suggest that the POMC precursor molecule undergoes similar processing in both lobes of the fetal pituitary, albeit to a different extent in each lobe and suggests that the intermediate lobe is a rich source of both ACTH and α-MSH during fetal life. The role of intermediate lobe ACTH in fetal life remains to be determined.

Chapter 8. Dopaminergic regulation of POMC gene expression in the ovine fetal pituitary gland

8.1. Introduction

The results presented in Chapter 6 clearly demonstrate the presence of a tonic dopaminergic inhibitory mechanism regulating the secretion of α-MSH and ACTH into the fetal circulation. However, analysis of pituitary peptide content (Chapter 7) suggests that the modified rate of peptide secretion in response to dopamine agonist or antagonist treatment does not influence the stored pituitary content of these peptides. Maintenance of pituitary peptide content occurring concomitantly with an altered rate of secretion may reflect regulation at the level of POMC gene expression.

The inhibitory influence of dopamine on POMC expression in the pituitary of adult rats has been extensively demonstrated. The capacity of the rat intermediate lobe to synthesise POMC, as measured by incorporation of [^{3}H] tyrosine was decreased by the dopamine agonist bromocriptine and increased by administration of the dopamine antagonist spiperidol (Beaulieu *et al.*, 1984). It has since been demonstrated that the altered level of POMC synthesis was reflected in parallel changes in the level of POMC mRNA in the intermediate lobe, with mRNA levels reduced by bromocriptine and increased by haloperidol (Chen *et al.*, 1983; Beaulieu *et al.*, 1984; Autelitano *et al.*, 1987; Chronwall *et al.*, 1987; Levy and Lightman, 1988). In all reports, the level of POMC gene expression in the anterior lobe was unaltered. Similar effects of bromocriptine and haloperidol have been demonstrated in primary cultures of isolated anterior and intermediate lobes of rat pituitary (Loeffler, Demeneix, Kley and Höllt, 1988). However, whilst the role of dopamine in the modulation of POMC gene expression in the rat pituitary has been studied by many workers, there is little information concerning dopaminergic regulation of POMC gene expression in sheep.

Thus, it was hypothesised that dopamine regulates POMC gene expression in the ovine fetal pituitary gland such that pituitary stores of POMC-derived peptides can be maintained during periods of enhanced or suppressed peptide secretion. To test this hypothesis, expression of POMC mRNA in the fetal pituitary in response to treatment with either the dopamine agonist bromocriptine or the dopamine

antagonist sulpiride was determined by in situ hybridisation. In addition, pituitary expression of the translated POMC products ACTH and α-MSH was determined by immunohistochemistry.

8.2. Materials and Methods

8.2.1. Animals and tissue collection

27 sheep of mixed breed with known insemination dates were used in this study. Fetal catheterisation was carried out at day 125 - 126 gestation (term =145 days) as described previously in Chapter 3. After overnight recovery the ewes were moved to metabolism crates and at least 5 days were allowed before experiments began.

8.2.2. Experimental Protocol

Beginning on day 131 gestation, fetuses received an intravenous infusion of either the dopamine antagonist sulpiride (0.3mg/0.5ml/hr ; n=8,), the dopamine agonist bromocriptine (0.03mg/0.5ml/hr ; n=3) or vehicle (0.1M tartaric acid in saline ; n=4) alone. Infusions commenced between 1100 and 1300 hours and continued for 72 hours. At the end of the infusion period, ewes were killed with an overdose of anaesthetic, fetuses delivered and fetal pituitaries collected as described previously (see Chapter 3). All isolated pituitaries were halved in a coronal plane before processing to paraffin wax and sectioning as described in section 3.6. In order to allow for comparison between gene expression and localisation of translated POMC-derived peptides consecutive sections were processed for in situ hybridisation and immunohistochemistry.

8.2.3. Radioactive in situ hybridisation for POMC

Radiolabelled riboprobe was prepared as described in section 3.11 and radioactive in situ hybridisation for POMC was carried out as described in section 3.12. For each pituitary in the study 8 tissue sections were mounted as 2 sections per slide on 4 separate slides and were treated with antisense riboprobe. 2 slides containing 2 tissue sections from each pituitary were treated with sense riboprobe as a negative control. Preliminary experiments revealed that POMC expression in pars distalis and pars intermedia was optimal at two different exposure times and so half of the antisense and sense hybridised slides were developed after 3 days for determination

of pars intermedia gene expression and the other half developed after 10 days for determination of pars distalis POMC gene expression.

8.2.4. Immunohistochemistry

Immunohistochemical detection of α-MSH and $ACTH_{(18-39)}$ was performed using the avidin-biotin staining procedure described in section 3.7.

8.2.5. Data analysis

All tissues in the study were processed together to allow for direct comparison between gestational ages. Each slide in the study contained a tissue section from an adult sheep pituitary as a positive control and to ensure standardisation of the procedure between slides. Sections were analysed using a computerised image analysis program (TCL-image, Multihouse, Amsterdam) for the Macintosh II computer. The position in the pituitary at which the analysis was undertaken was consistent throughout. As POMC gene expression in the pars distalis is regionally distributed, the area around the intermediate lobe of the pituitary and the area at the base of the anterior lobe were analysed separately. A minimum of 3 separate tissue sections were analysed from each pituitary in the study and 5 fields were counted for each area in the tissue to be analysed.

8.3. Results

Localisation of POMC mRNA in a representative pituitary section from each treatment group is shown in Figure 8.1. POMC mRNA was abundantly expressed in the intermediate and anterior lobes of the pituitary in all three groups. The regional distribution of POMC mRNA was unaffected by either bromocriptine or sulpiride treatment when compared to vehicle-treated controls.

Computerised image analysis of silver grains revealed that the POMC mRNA in the intermediate lobe of the pituitary was significantly ($p<0.05$) decreased following bromocriptine treatment (Figure 8.2a). There was no significant effect of sulpiride on POMC mRNA expression in the intermediate lobe. POMC expression in both the basal region of the anterior pituitary and the region surrounding the intermediate lobe was not significantly altered by treatment with either bromocriptine or sulpiride (Figure 8.2b).

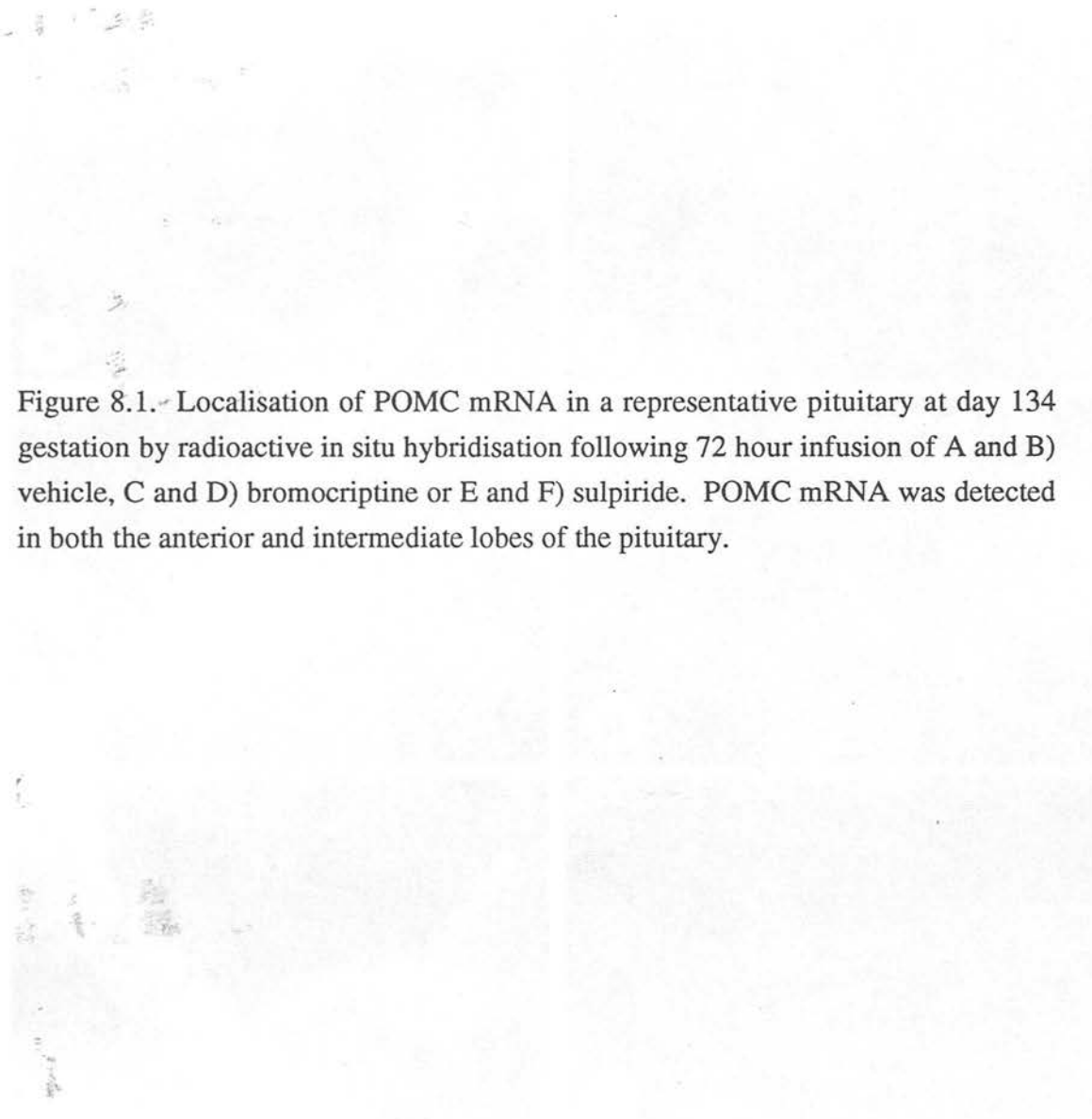
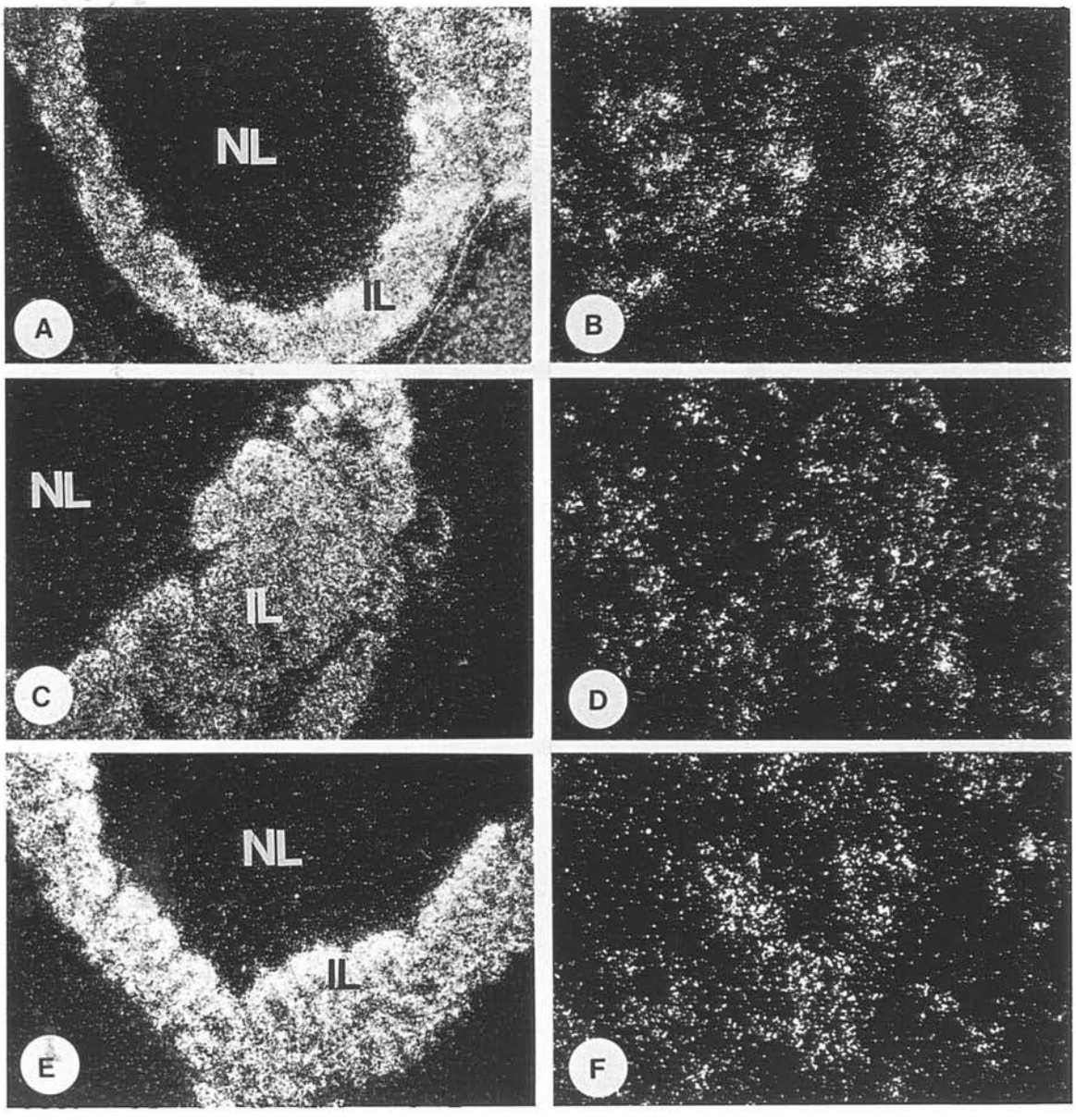


Figure 8.1. Localisation of POMC mRNA in a representative pituitary at day 134 gestation by radioactive in situ hybridisation following 72 hour infusion of A and B) vehicle, C and D) bromocriptine or E and F) sulpiride. POMC mRNA was detected in both the anterior and intermediate lobes of the pituitary.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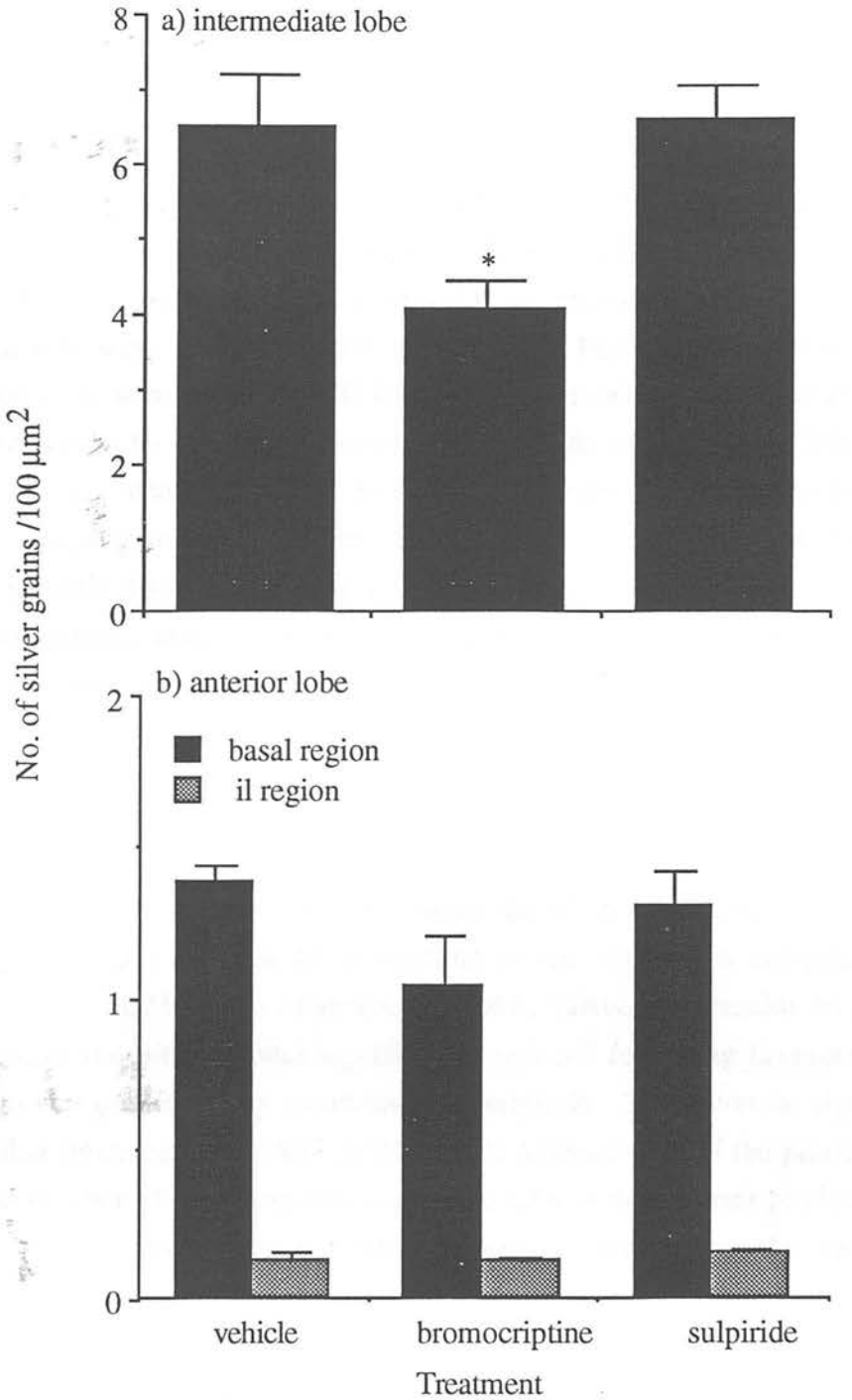


Figure 8.2. Histogram showing the number of silver grains per 100 square microns of tissue in a) the intermediate lobe and b) the anterior lobe of the pituitary. In the anterior lobe, the region at the base of the gland (black bars) and the region immediately adjacent to the intermediate lobe (grey bars) were analysed separately. Significant difference from corresponding vehicle control is indicated by an asterisk, * $P<0.05$.

However, the levels of POMC mRNA in the basal region of the anterior pituitary tended to decrease in response to bromocriptine when compared to vehicle treated controls (Figure 8.2b; p=0.09)

Immunohistochemical detection of α-MSH and ACTH in a representative pituitary section from each treatment group are shown in Figures 8.3 and 8.4 respectively. α-MSH immunoreactivity was confined to the intermediate lobe and almost all cells of the lobe were immunopositive (Figure 8.3). For this reason it was not possible to count the number of α-MSH immunopositive cells. The intensity of staining was not altered by either bromocriptine or sulpiride treatment. ACTH immunopositive cells were identified in both the anterior and intermediate lobe of the pituitary gland. In keeping with the pattern of POMC gene expression, ACTH-ir cells were regionally distributed with the region of the anterior lobe closest to the intermediate lobe largely devoid of corticotrophs (Figure 8.4). The number of immunopositive corticotrophs in the corticotroph dense region around the base of the gland was unaltered by treatment with bromocriptine or sulpiride (Figure 8.5).

8.4. Discussion

The aim of this study was to determine the effect of a 72 hour intrafetal infusion of the dopamine agonist bromocriptine or the dopamine antagonist sulpiride on pituitary POMC gene expression. POMC mRNA expression in the intermediate lobe of the pituitary was significantly reduced following bromocriptine treatment and was unaffected by treatment with sulpiride. There was no significant effect of either treatment on POMC mRNA in the anterior lobe of the pituitary. In addition, the number of immunopositive corticotrophs in the anterior pituitary was unaltered by either bromocriptine or sulpiride treatment when compared to vehicle controls.

The decreased POMC mRNA levels in the intermediate lobe following bromocriptine treatment reported in the present study are consistent with previous reports in the adult rat (Chen *et al.*, 1983; Beaulieu *et al.*, 1984; Autelitano *et al.*, 1987; Chronwall *et al.*, 1987; Levy and Lightman, 1988). In all of the studies in rats, the fall in POMC mRNA in the intermediate lobe was greater than that described here (50-80%). However, the dose of bromocriptine used was typically 5-10 fold greater than that used in the present study. This suggests that the suppression of POMC mRNA levels induced by bromocriptine treatment in

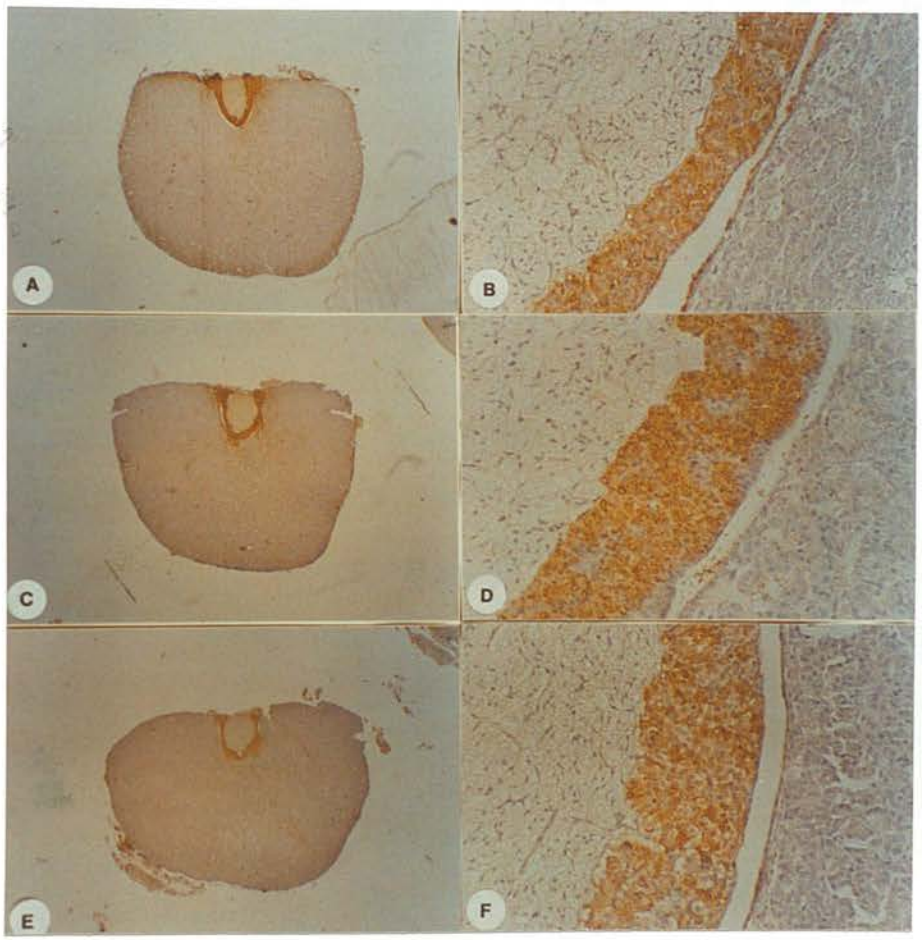


Figure 8.3. Immunohistochemical detection of α-MSH in a representative pituitary section following 72 hour treatment with (A, B) vehicle, (C, D) bromocriptine or (E, F) sulpiride. α-MSH immunostaining was confined to the intermediate lobe. The distribution and intensity of staining was affected by treatment of bromocriptine or sulpiride when compared to vehicle treated controls. A, C and E x1 magnification, B, D and F x20 magnification.

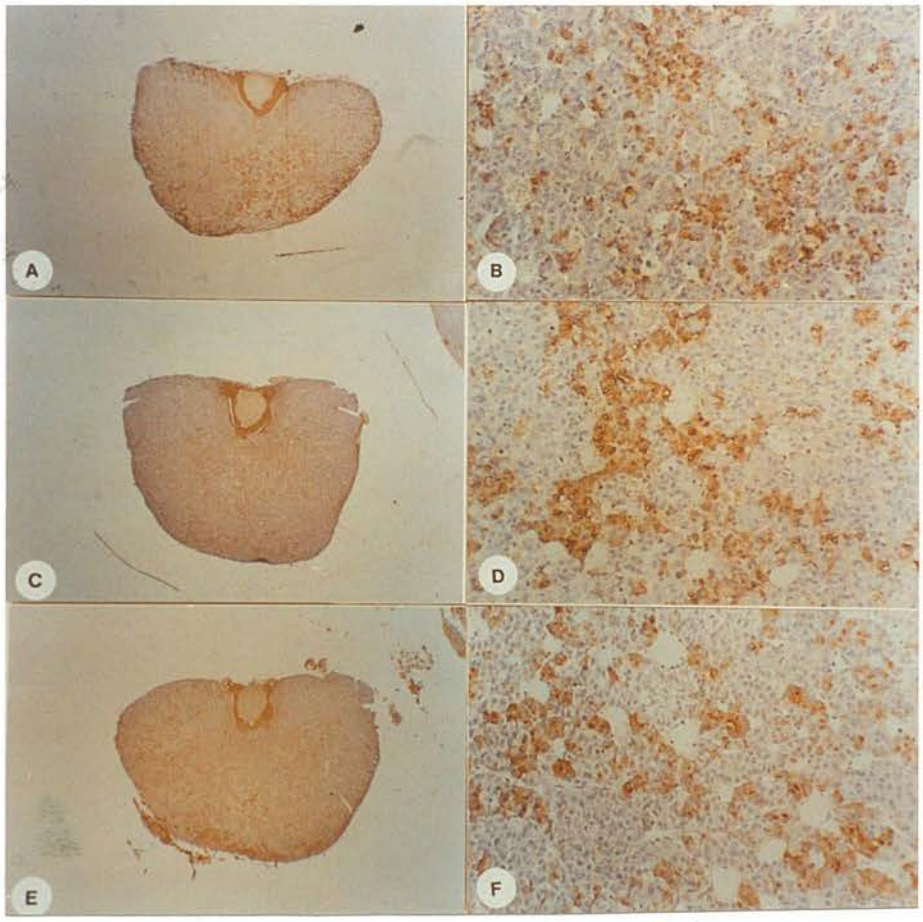


Figure 8.4. Immunohistochemical detection of $ACTH_{(18-39)}$ in a representative pituitary section following 72 hour treatment with (A, B) vehicle, (C, D) bromocriptine or (E, F) sulpiride. ACTH immunoreactive cells were identified in both the anterior and intermediate lobes of the pituitary. Anterior pituitary corticotrophs tended to be regionally distributed with the majority of immunopositive cells occupying the basal aspect of the gland. A, C and E x1 magnification, B, D and F x20 magnification.

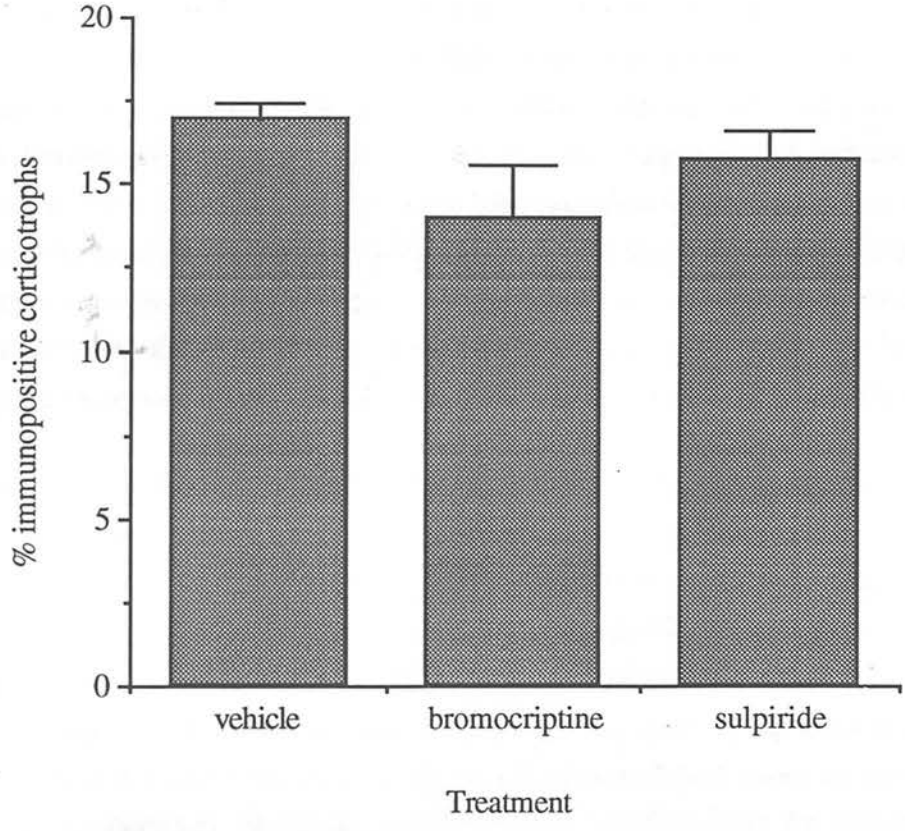


Figure 8.5. The percentage of immunopositive corticotrophs in the basal aspect of the anterior pituitary in fetuses treated for 72 hours with vehicle, bromocriptine or sulpiride expressed as a percentage of the total number of cells present. The percentage of immunopositive corticotrophs was unaffected by infusion of bromocriptine or sulpiride when compared to vehicle treated controls. Data shown represent the mean ± SEM for all fetuses in each treatment group.

the present study is perhaps not maximal and could be enhanced by increased doses of bromocriptine. Dose-response studies would be needed to clarify this point.

Chronwall *et al.* (1987) reported that autoradiographic grains over intermediate lobe melanotrophs were heterogeneously distributed, indicating that different cells maintained different levels of POMC gene expression. The decrease in POMC mRNA levels following acute bromocriptine treatment occurred concomitantly with a diminished heterogeneity of grain distribution, suggestive of differing responses of individual cells. Following chronic administration of bromocriptine, the rate of cell proliferation was altered, as determined both by incorporation of [^{3}H]thymidine and measurements of mitotic index. This data suggests that the decrease in POMC mRNA levels in response to bromocriptine results from a change in both the level of gene expression and in the number of melanotrophs present in the intermediate lobe.

A decreased level of POMC mRNA in the fetal intermediate lobe following bromocriptine may represent either a decrease in synthesis and processing of the POMC mRNA precursor molecule or increased translation of the mature mRNA. In the present study it is likely that the decrease in POMC mRNA levels represents a decreased rate of POMC mRNA synthesis as there was no concomitant increase in pituitary content of POMC-derived peptides (Chapter 7). In addition, an increased rate of translation with no increase in cell content would imply an increased level of peptide secretion. However, the secretion of α-MSH from the intermediate lobe to the fetal circulation is reduced following bromocriptine treatment (Chapter 6).

In the present study there was no effect of sulpiride on intermediate lobe POMC mRNA expression. This contrasts considerably with data generated in adult rats in which the dopamine antagonist haloperidol has been demonstrated to significantly increase the level of POMC mRNA in the intermediate lobe of the pituitary (Chen *et al.*, 1983; Autelitano *et al.*, 1987; Chronwall *et al.*, 1987). The lack of effect of sulpiride on POMC mRNA levels in the present study may simply be due to the fact that the mechanisms controlling POMC expression are fundamentally different in the rat and sheep or that the mechanisms are sufficiently different in the adult compared to the fetus. It is not possible to rule out these potential factors completely. However, an alternative explanation lies in the already very high level of expression of POMC mRNA in the fetal intermediate lobe. Levels of POMC mRNA are consistently high in the intermediate lobe, indeed they are significantly

greater than those in the anterior lobe at any point in late gestation (Matthews *et al.*, 1994; Chapter 5). Consequently it is possible that the gene is maximally expressed during late gestation and as such can not be further stimulated by dopamine blockade. If this is the case, factors other than increased gene expression must be acting to account for the increased levels of POMC-derived peptides present in the fetal circulation and the maintenance of intra-pituitary peptide content following sulpiride treatment. One likely mechanism by which this could occur is by regulation of the post-translational processing of the POMC protein precursor molecule. Support for this hypothesis comes from the demonstration that the post-translational processing of β-endorphin in the rat intermediate pituitary is modified by dopamine (Millington *et al.*, 1987). Long-term treatment of rats with either dopamine agonists or antagonists selectively altered the molecular forms of β-endorphin contained in and released from the intermediate lobe (Millington *et al.*, 1987). Thus, it is possible that sulpiride influences the post-translational processing of existing stores of POMC in the intermediate lobe to maintain the increased demand for secreted peptide. As discussed in Chapter 7, increased POMC mRNA expression following dopamine antagonist treatment in adult rats is accompanied by increased levels of PC1 and PC2 mRNA, with PC2 mRNA levels showing the greatest increase (Day *et al.*, 1992). It would be very interesting to investigate the levels of PC1 and PC2 expression in the present system as altered post-translational processing of the POMC molecule to favour production of α-MSH from the intermediate lobe would facilitate increased α-MSH production from a steady state level of POMC gene expression. Thus, POMC mRNA levels in the intermediate lobe of the pituitary need not necessarily increase to fulfil the demands of augmented peptide secretion.

The lack of effect of bromocriptine and sulpiride on anterior pituitary POMC mRNA expression is consistent with studies in the adult rat (Chen *et al.*, 1983; Levy and Lightman, 1988). However, there was a tendency towards a decrease in POMC mRNA levels in the basal aspect of the anterior pituitary, although this decline did not reach significance. The lack of a significant effect on anterior pituitary POMC gene expression is less surprising since there is little evidence to suggest that anterior pituitary corticotrophs are regulated by dopamine. In contrast to the well documented regulation of the intermediate lobe of the pituitary by dopamine, the anterior pituitary is primarily under the influence of the hypothalamic neuropeptides CRH and AVP (Challis and Brooks, 1989). In the late gestation fetal sheep there is

a concomitant rise in the levels of POMC mRNA in the anterior pituitary and that of CRH mRNA levels in the paraventricular nucleus of the hypothalamus (Myers *et al.*, 1993). As bromocriptine is administered intravenously it is likely that it has several sites of action. Thus, it is possible that bromocriptine treatment acts indirectly on the hypothalamus to reduce levels of CRH mRNA which in turn may precipitate a decline in POMC mRNA in the anterior pituitary. To this end, levels of POMC mRNA in the anterior pituitary of adult rats have been shown to increase significantly in response to 15 day administration of CRH (Bruhn *et al.*, 1984). It remains possible that, following a longer treatment period with bromocriptine, expression of POMC mRNA in anterior pituitary corticotrophs of the ovine fetus may be significantly reduced. As the number of anterior pituitary corticotrophs was unaltered by infusion of either bromocriptine or sulpiride it seems likely that any influence of bromocriptine on POMC mRNA in the anterior pituitary, occurring either directly or indirectly, reflects a down-regulation in the expression levels of individual cells rather than an effect on the number of cells actively expressing the POMC gene within the corticotroph population.

In conclusion, this study demonstrated that dopaminergic modulation of the fetal intermediate lobe can occur at the level of gene expression. Moreover, it suggests that mechanisms other than modification in the rate of gene expression, such as post-translational processing are important in the pituitary response to dopaminergic blockade by sulpiride.

Chapter 9. Dopaminergic regulation of fetal prolactin secretion

9.1. Introduction

Previous studies in the ovine fetus have demonstrated that plasma prolactin concentrations are modulated by dopamine. Administration of the dopamine agonists bromocriptine or apomorphine by intravenous infusion or bolus injection can suppress circulating plasma prolactin concentrations in fetal sheep (Gluckman *et al.*, 1979; Lowe, Beck, McNaughton, Gluckman, Kaplan, Grumbach and Nathanielsz, 1979) and in neonatal lambs (Gluckman *et al.*, 1979). Conversely, administration of the dopamine antagonist haloperidol acts to stimulate the release of prolactin into the fetal circulation in fetuses from day 106 gestation, demonstrating the presence of a tonic inhibitory dopaminergic mechanism regulating the secretion of prolactin in the developing fetal sheep (Gluckman *et al.*, 1979).

Prolactin concentrations in adult sheep are influenced by season such that circulating concentrations are high during the long days of the summer months and low during the short days of the winter months (Curlewis, 1992). A daily rhythm in prolactin concentrations has also been demonstrated, with a peak of plasma prolactin concentrations associated with the onset of darkness (Brinklow and Forbes, 1984; Walton, Evins, Fitzgerald and Cunningham, 1989). Recently it has become clear that fetal plasma prolactin concentrations are also influenced by the duration of the external photoperiod (Basset, Bomford and Mott, 1988). Fetal plasma displays a diurnal rhythm of both prolactin (McMillan, Thorburn and Walker, 1987) and melatonin (Zembegs, McMillen, Walker, Thorburn and Nowak, 1988) which reflects that found in the maternal circulation. Experimental manipulation of the photoperiod experienced by the ewe confirmed that photoperiodic information transmitted from the ewe to the fetus influences fetal plasma prolactin concentrations (Bassett, Curtis, Hanson and Weeding, 1989). Fetal plasma prolactin concentrations can be suppressed under long days by maternal infusion of melatonin (Bassett *et al.*, 1989), demonstrating that the nocturnal increase in plasma melatonin in the ewe is instrumental in the release of fetal prolactin. Moreover, pinealectomy of the ewe abolishes the diurnal rhythm of melatonin in both the ewe and the fetus (Yellon and Longo, 1989; McMillen and Nowak, 1989).

It has been suggested that the response of the ovine fetal pituitary to the prolactin releasing factor thyrotropin-releasing factor (TRF) increases with advancing gestational age (Thomsett, Marti-Henneberg, Gluckman, Kaplan, Rudolph and Grumbach, 1980). Similarly, Gluckman *et al.* (1983) report an increase in response of the fetal pituitary to a bolus injection of the dopamine antagonist haloperidol with advancing gestation. More recently, Bassett *et al.* (1989) report that fetal response to TRH and the dopamine antagonist metoclopramide are primarily determined by photoperiod rather than ontogenic development.

The aim of this study was to determine the extent to which season influences the dopaminergic regulation of prolactin secretion in the late gestation ovine fetus. Specifically, fetuses at day 131 gestation were treated with 72 hour intravenous infusion of either bromocriptine or sulpiride between January and March or between May and July, and the response in terms of fetal plasma prolactin concentrations was determined.

9.2. Materials and Methods

9.2.1. Experimental protocol

Fetuses were prepared with indwelling femoral catheters at day 125-126 gestation as described in section 3.2. The experiment consisted of two groups of animals. One experiment was performed between January and March (n=12; hereafter called the winter group) and the other was performed between May and July (n=11; hereafter called the summer group). Beginning on day 131 gestation, fetuses received a 3 day intravenous infusion of either bromocriptine (0.03mg/0.5ml/hr) or sulpiride (0.3mg/0.5ml/hr) as described previously (section 6.2.2). Fetal blood samples (1ml) were collected at 12 hour intervals (0900 and 2100 hours) beginning 24 hours before the start of the infusion for the determination of plasma prolactin concentrations by radioimmunoassay (section 3.4.2). At the end of the infusion period, ewes were killed and fetal pituitaries were collected into 4% paraformaldehyde for prolactin immunohistochemistry (section 3.6). Pituitaries collected during the summer months were collected and fixed intact whilst those collected during the winter months were dissected to remove the intermediate lobe for the study of peptide content described in Chapter 7. In these fetuses, a 2-3mm thick coronal slice of anterior pituitary was removed from the middle of the pituitary gland following

dissection of the intermediate lobe, fixed in 4% paraformaldehyde and utilised for prolactin immunohistochemistry.

The percentage of lactotrophs in the pituitary was determined by counting the number of cells immunopositive for prolactin relative to the total number of cells present in the anterior lobe of the pituitary. Lactotrophs tended to be regionally distributed with less cells present in the central region of the anterior lobe. Four fields were counted in the lactotroph dense region of the anterior lobe in each tissue section and at least two tissue sections were counted for each pituitary in the study.

9.2.2. Data analysis

The effects of bromocriptine and sulpiride infusion on plasma prolactin concentrations over the 72 hour period was analysed by Analysis of Variance (ANOVA) with repeated measures using the Statview package (Abacus Concepts, Inc., Berkeley, Ca , 1992) for the Apple Macintosh. Comparison of means was analysed using Students t-test where indicated. All data shown are mean ± SEM and in all analysis, $p<0.05$ was considered to be significant.

9.3. Results

9.3.1. Plasma concentrations

Basal prolactin concentrations in fetuses during winter and summer months are shown in Figure 9.1. Data shown represent the mean plasma prolactin concentration in the three daily blood samples withdrawn from all fetuses treated in winter (n=12) and summer (n-11) prior to the start of the experimental infusion. Mean measured plasma prolactin concentrations were significantly ($p<0.001$; Students t-test) higher in summer fetuses (127.61 ng/ml ± 8.26, mean ± SEM) when compared to winter fetuses (6.28 ± 0.49).

The plasma prolactin concentrations in representative fetuses infused with vehicle, bromocriptine or sulpiride are shown in Figure 9.2 a, b and c respectively. Profiles in the upper panel were generated during the summer months whilst those in the lower panel represent fetuses treated during the winter. In both seasons, plasma

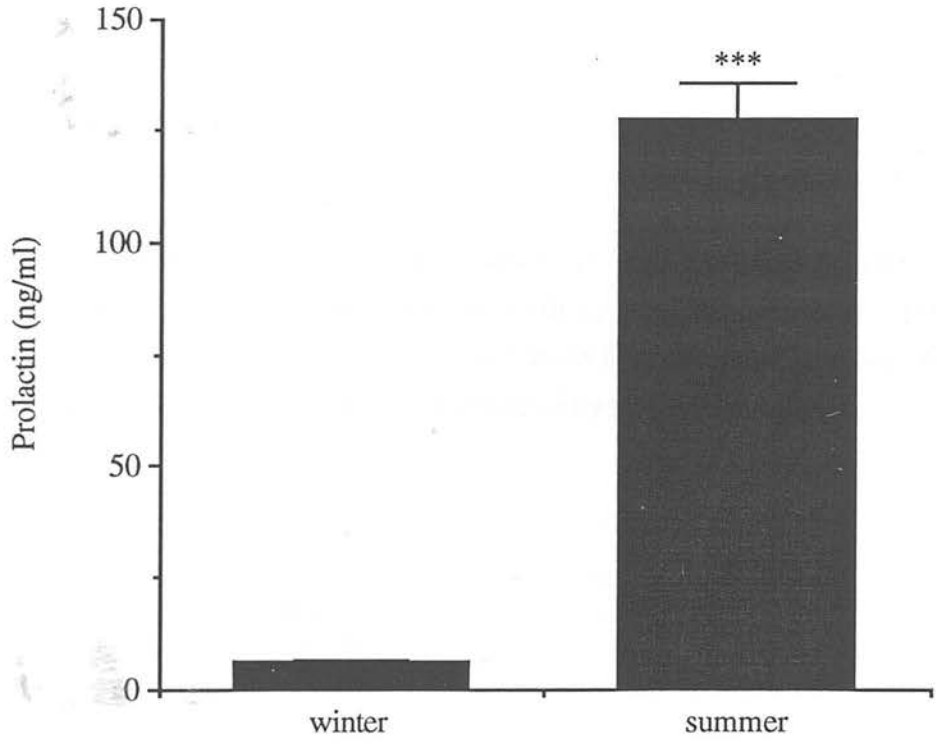
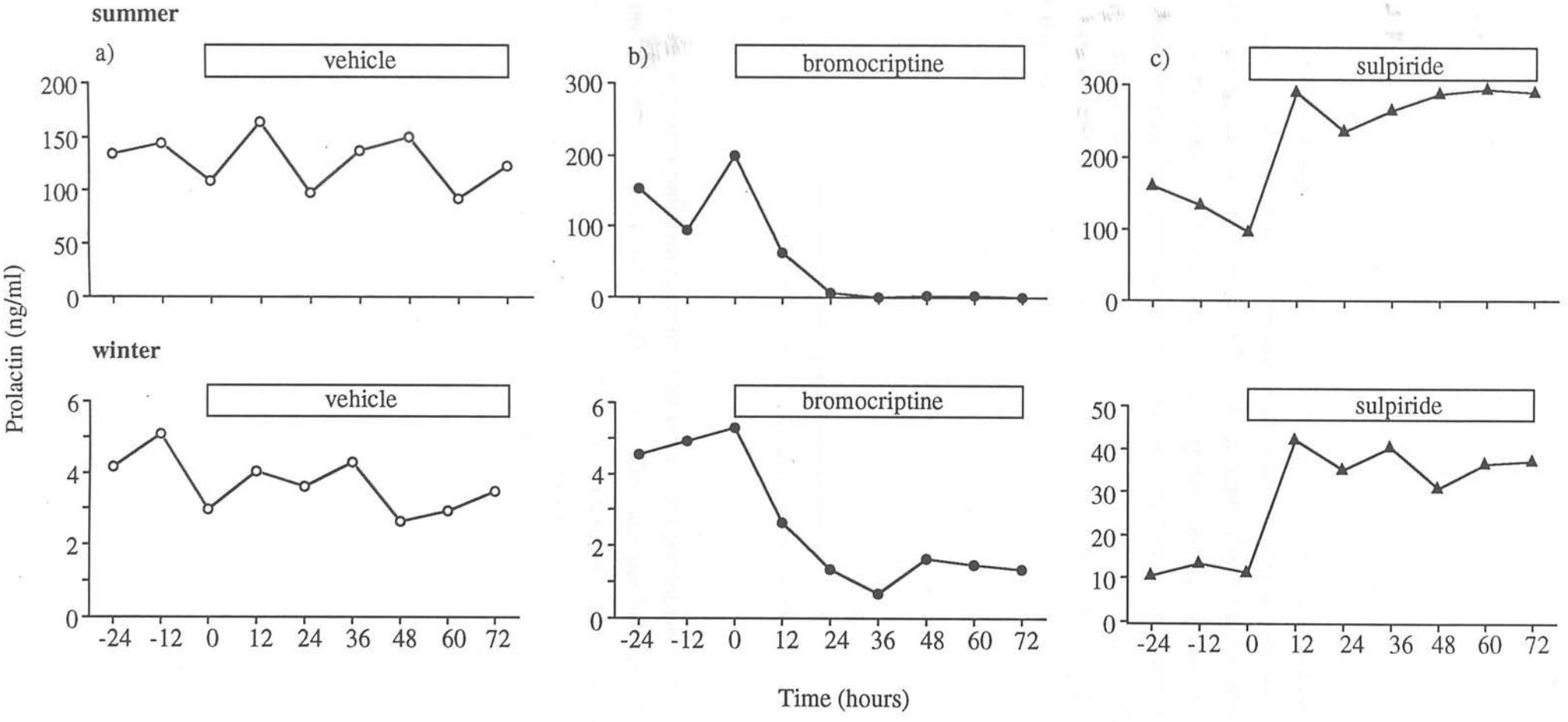


Figure 9.1. Mean plasma prolactin concentration in fetuses sampled prior to the start of the infusion, between January and March (winter ; n=12) and May and July (summer ; n=11). There was a highly significant (***, $p<0.001$) difference between winter and summer basal prolactin concentrations

Figure 9.2. Plasma prolactin concentrations in blood samples collected every 12 hours in representative fetuses infused with a) vehicle, b) bromocriptine and c) sulpiride. Profiles in the upper panel are from fetuses infused during the summer and those in the lower panel are from fetuses infused in the winter.


summer
a)
vehicle
b)
bromocriptine
c)
sulpiride
winter
vehicle
bromocriptine
sulpiride
Prolactin (ng/ml)
Time (hours)
-24 -12 0 12 24 36 48 60 72

prolactin concentrations were unaffected by infusion of vehicle. Infusion of bromocriptine (9.2b) precipitated a steady decline in prolactin concentrations in both summer and winter fetuses. Conversely, sulpiride infusion (9.2c) induced a rapid increase in circulating prolactin concentrations irrespective of season.

The mean fetal plasma prolactin response of all fetuses in each treatment group to a 72 hour infusion of vehicle control, bromocriptine or sulpiride in both the summer and winter months is shown in Figure 9.3. In both summer (9.3a) and winter (9.3b) fetuses, plasma prolactin concentrations were significantly ($p< 0.001$) increased above basal values following sulpiride infusion and levels were significantly ($p<0.001$ and $p<0.01$; summer and winter respectively) reduced following bromocriptine treatment. Infusion of sulpiride resulted in a rapid increase in circulating prolactin concentrations in both winter and summer fetuses which was maximal at least 12 hours after the start of the infusion. By contrast, the response to bromocriptine at both times of year was more gradual, with plasma concentrations steadily declining over a 24-36 hour period and then remaining consistently low for the remainder of the infusion period. The response of winter fetuses to bromocriptine infusion is not easily discernible on the scale of Figure 9.3a due to the large increase in concentrations following sulpiride infusion. However, the fall in prolactin levels in response to bromocriptine is clearly shown in Figure 9.2b (lower panel).

The change in plasma prolactin concentration from basal pre-infusion values over the 72 hour infusion period is shown in Figure 9.4. The basal pre-infusion value was calculated by averaging the three daily blood samples withdrawn prior to the start of the experimental infusion and the mean prolactin concentration in samples collected during the 72 hour infusion period were expressed as a change from the pre-infusion value. Whilst treatment of fetuses with sulpiride during the summer months resulted in the greatest absolute plasma prolactin concentrations (Figure 9.3), the change from baseline concentrations was similar for both summer and winter fetuses. Winter fetuses displayed a 2.7 fold increase in prolactin concentrations compared to the 2.2 fold increase over basal prolactin concentrations in summer fetuses.

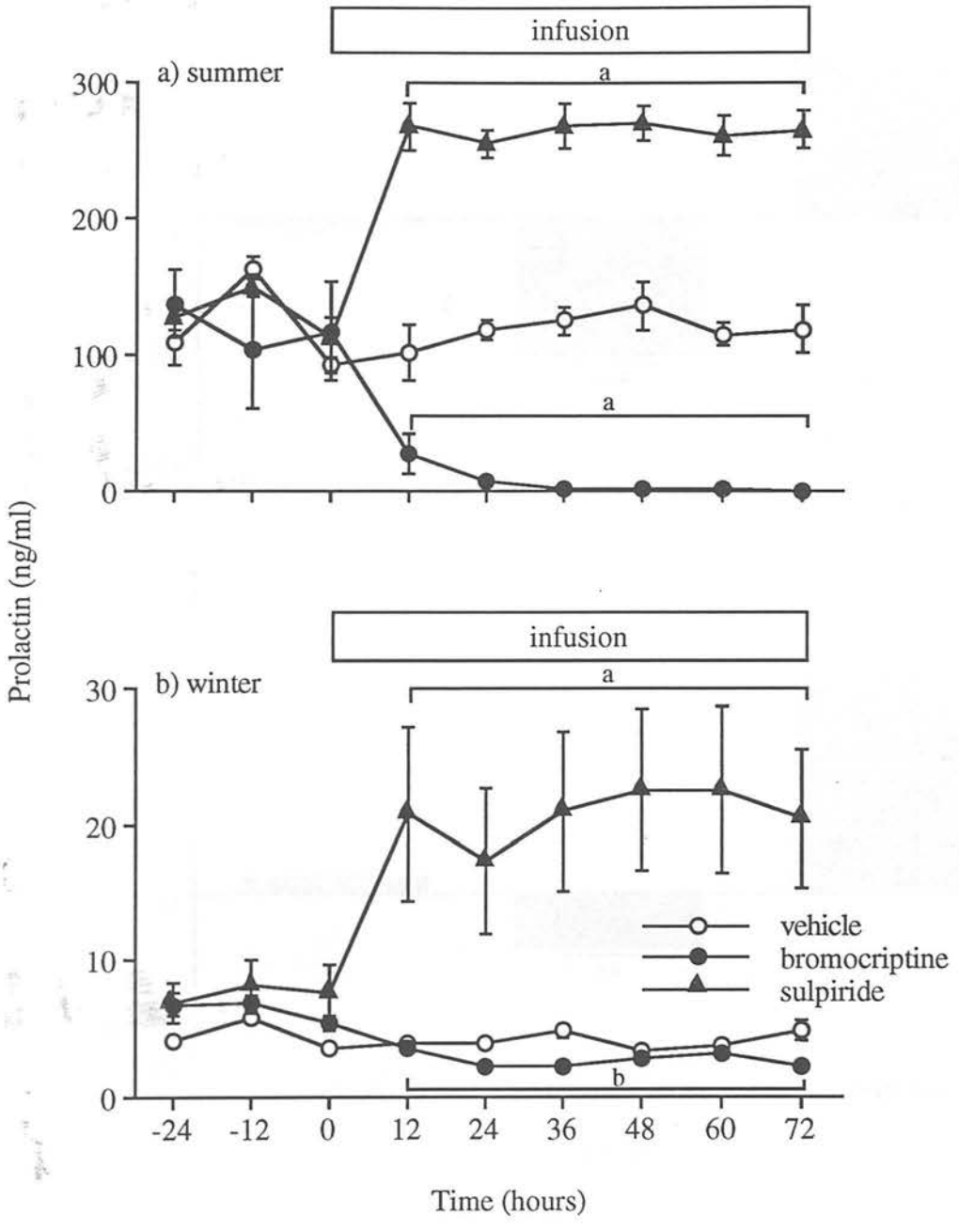


Figure 9.3. Change in fetal plasma prolactin concentrations in a) summer and b) winter fetuses infused with vehicle, bromocriptine or sulpiride. Data shown represent the mean concentrations at 12 hour intervals from all fetuses in each treatment group. Significant differences from corresponding controls are indicated by a, $p < 0.001$;b, $p \leq 0.01$. Note the different scale on the y-axis for summer and winter periods.

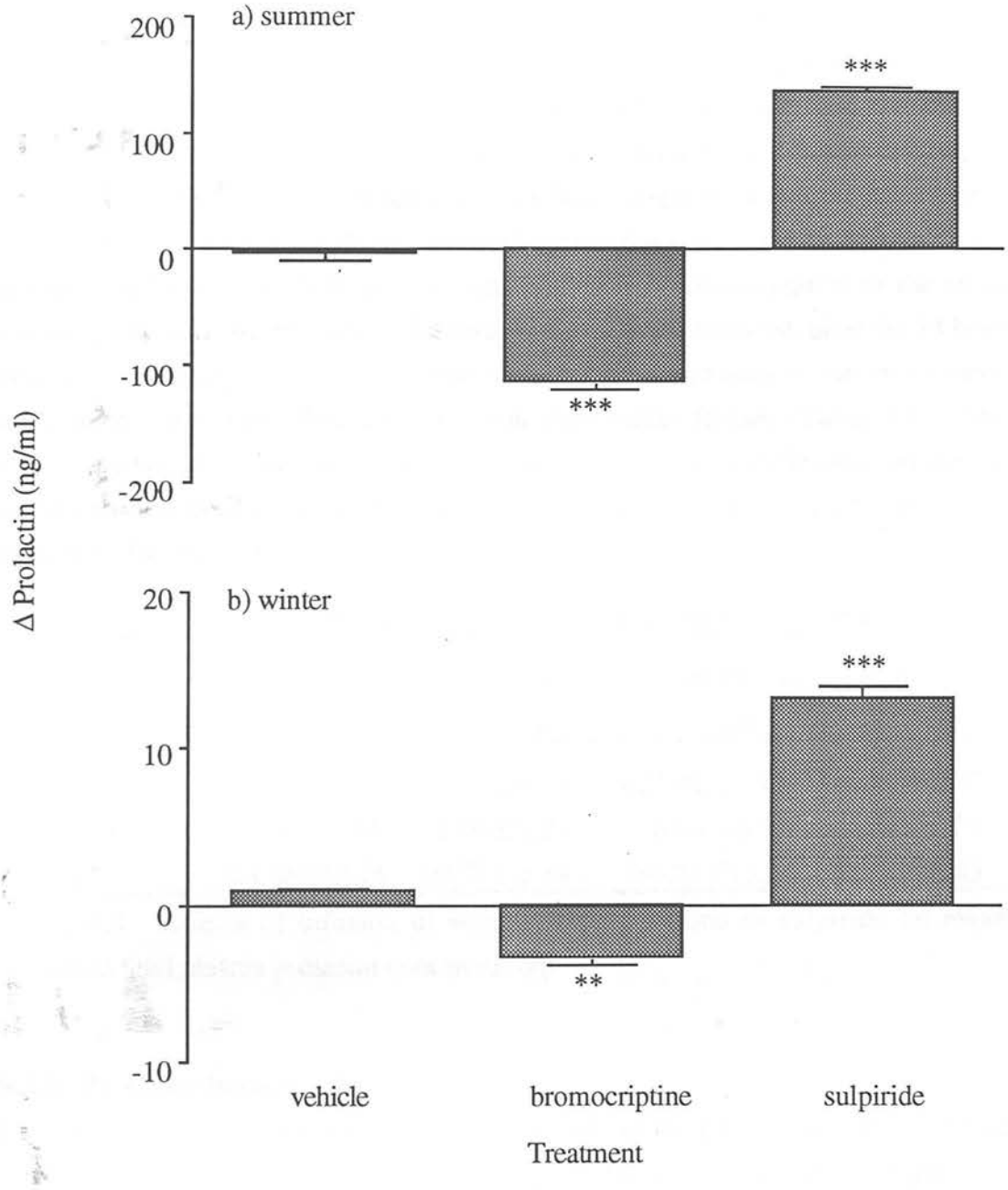


Figure 9.4. Change in prolactin concentrations from basal values in a) summer and b) winter fetuses averaged over the entire 72 hour infusion period. Significant differences from corresponding vehicle treated controls are indicated by asterisks (** $p<0.01$; *** $p< 0.001$).

Although sulpiride treatment significantly increased plasma prolactin concentrations in winter fetuses, the maximum prolactin concentration remained considerably lower than basal levels of vehicle-treated control fetuses in the summer period (Table 9.1). Mean plasma prolactin concentrations during the 72 hour bromocriptine infusion in summer treated fetuses did not reach the low basal levels of winter control fetuses. However, the fall in prolactin concentration from the high summer values occurs gradually over the first 24 hour of the infusion period. Determination of the mean plasma prolactin concentration in the final 48 hours of the infusion, after the 24 hour period of declining values, reveals that prolactin concentrations in summer fetuses are reduced to a lower final concentration than winter fetuses (Table 9.1). The multiplicative response to bromocriptine treatment was significantly greater in fetuses treated during the summer months (18 fold reduction) compared to winter months (2 fold reduction).

	Mean plasma prolactin concentration (ng/ml)			
	complete 72 hour infusion		last 48 hours of infusion	
Treatment	summer	winter	summer	winter
vehicle	119.63 ± 6.64	4.12 ± 0.26	124.08 ± 5.73	4.22 ± 0.37
bromocriptine	6.56 ± 2.51	2.86 ± 0.91	0.96 ± 0.07	2.85 ± 0.28
sulpiride	264.86 ±12.75	20.77 ± 5.44	266.53 ±13.24	21.62 ± 5.43

Table 9.1. Effects of infusion of vehicle, bromocriptine or sulpiride on mean measured fetal plasma prolactin concentration.

9.3.2. Prolactin immunostaining

Immunohistochemical identification of lactotrophs in the pars distalis revealed that lactotrophs tended to occupy the lateral wings of the anterior pituitary. Figure 9.5 shows representative anterior pituitary sections from summer (9.5a) and winter (9.5b) fetuses which were immunostained for prolactin. Cell counts of prolactin immunopositive cells in the lactotroph dense regions revealed that there was a significantly ($p < 0.001$) smaller percentage of immunopositive lactotrophs during the winter months (15.01 ± 0.7%) when compared to the summer months (21.52 ± 0.5%, Figure 9.6a). The percentage of immunopositive lactotrophs was unaffected by infusion of bromocriptine or sulpiride in either the summer or winter season (Figure 9.6b).

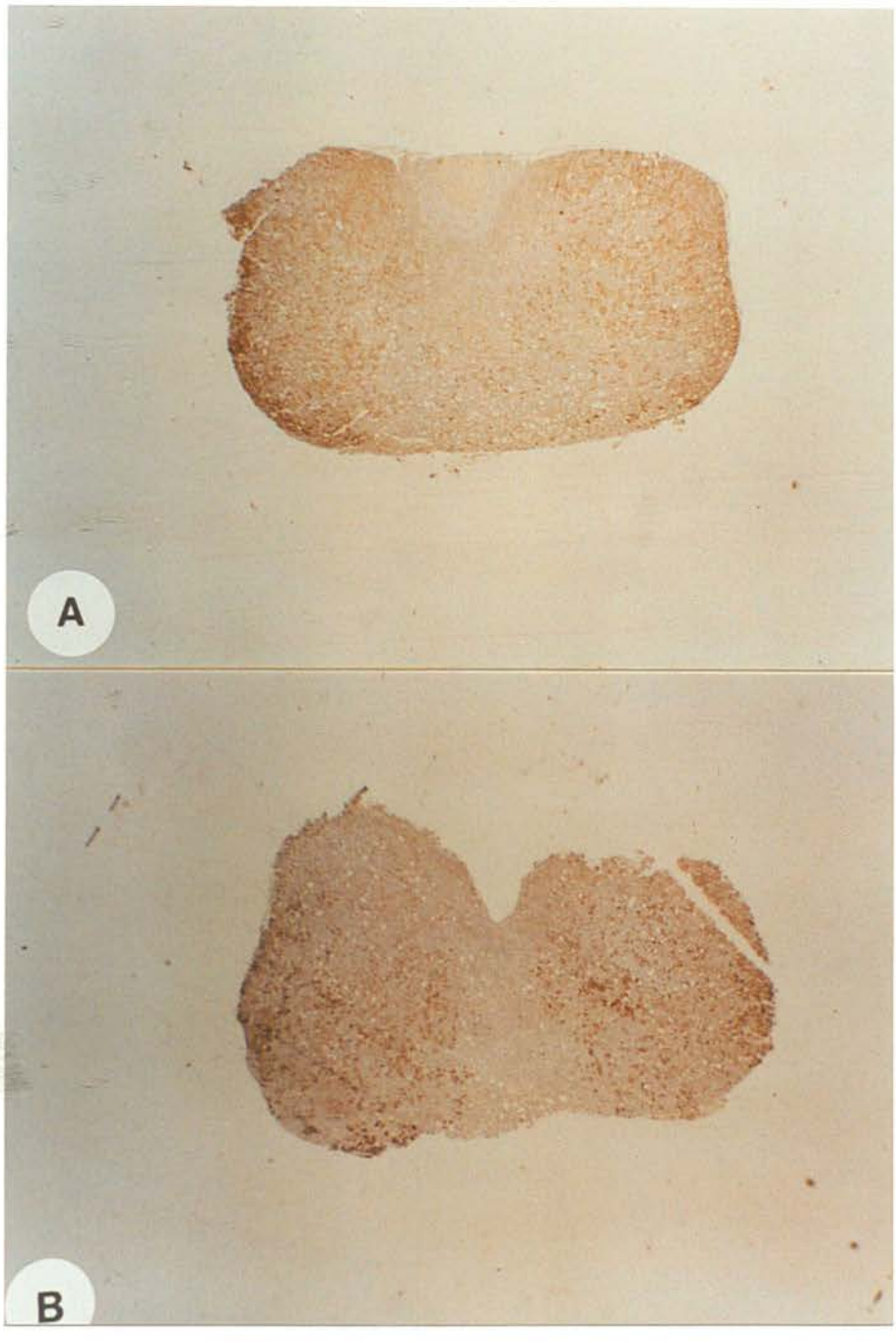


Figure 9.5. Immunohistochemical localisation of prolactin in representative pituitary sections collected from a summer (a) and a winter (b) fetus. Lactotrophs were regionally distributed with less immunostaining present in the central region of the pars distalis.

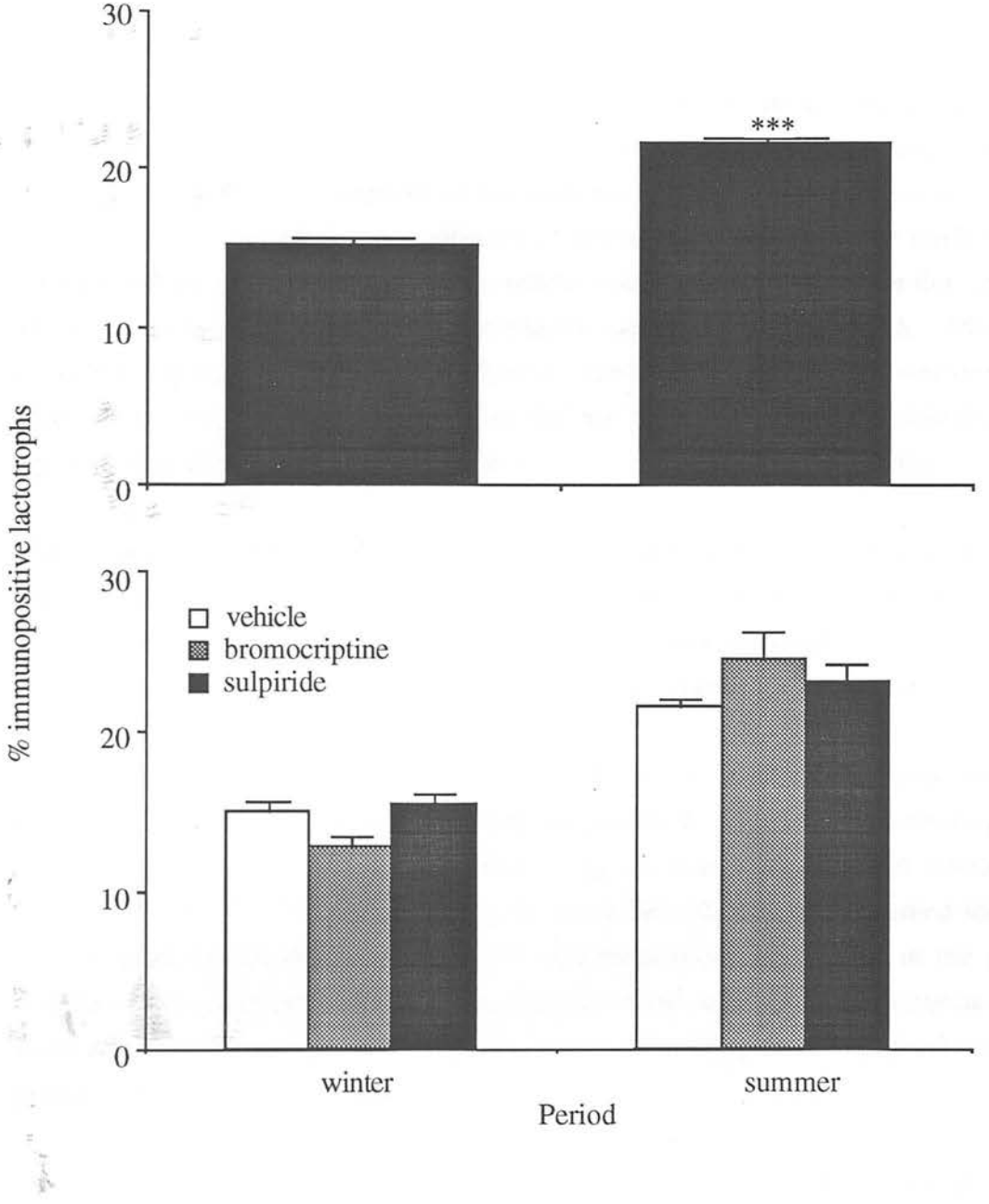


Figure 9.6. a) The percentage of prolactin immunopositive cells in the lateral region of the pars distalis of vehicle-infused fetuses treated in summer (n=4) and winter (n=3). There was a significant (*** $p<0.001$) difference in the number of lactotrophs between summer and winter. b) The percentage of immunopositive lactotrophs was unaffected by infusion of bromocriptine or sulpiride in summer or winter.

9.4. Discussion

Previous studies have reported that prolactin secretion in the ovine fetus is modulated both by dopamine and by external photoperiod. The present study investigated the prolactin response to dopaminergic modulation in different seasons. Fetuses received an intravenous infusion of bromocriptine or sulpiride during the winter (January - March), when basal prolactin concentrations are low or during the summer (May - July), when basal prolactin concentrations are high. Plasma prolactin concentrations were significantly elevated in response to infusion of sulpiride in both seasons, demonstrating that the tonic dopaminergic inhibition of prolactin secretion is present during both the winter and the summer months.

The mean concentration of prolactin in the fetal plasma was significantly greater in the summer than in the winter. This is in accordance with the previously reported influence of external photoperiod on fetal plasma prolactin levels (Bassett *et al.*, 1988). Bassett and co-workers measured prolactin concentrations in late gestation fetal and adult sheep during hormonally-extended breeding seasons from October to June and demonstrated that the level of prolactin in fetal plasma was closely correlated with the variation in ambient photoperiod, with levels increasing as daylength increased. The present study suggests that the decreased levels of prolactin in the fetal circulation during the winter months can be accounted for, at least in part, by the reduced number of immunopositive lactotrophs in the pars distalis of the pituitary in the winter as opposed to the summer. This suggests that there are fewer functional lactotrophs actively contributing to the basal release of prolactin during the winter.

In the present study, the highest concentrations of prolactin were achieved after sulpiride treatment of fetuses during the summer. Whilst dopamine receptor blockade during the winter months resulted in a rapid increase in circulating prolactin concentrations, plasma prolactin levels remained considerably lower than basal levels of vehicle-treated control fetuses in the summer period. This finding is at odds with the proposal that low plasma prolactin concentrations during short day photoperiods are a result of increased dopaminergic inhibition (Curlewis, 1992). Whilst it is clear that dopamine acts to suppress the level of prolactin in winter fetuses it is clearly not solely responsible since levels remain low following

dopamine receptor blockade. The results reported in this chapter are consistent with the findings of Bassett *et al.* (1989) who reported that neither TRH nor the dopamine antagonist metoclopramide was able to increase plasma prolactin concentrations of winter lambs to levels experienced by summer lambs.

The inability of the winter pituitary to secrete prolactin in response to sulpiride at concentrations that are comparable with basal levels from summer fetuses could be a consequence of the reduced number of lactotrophs in the winter. As well as limiting the amount of prolactin available for basal secretion, the reduced number of lactotrophs in the winter pituitary are likely to limit the extent to which the pituitary can respond to dopamine receptor blockade. Despite the great difference in maximum measured concentration of prolactin in response to sulpiride infusion in winter and summer fetuses, the scale of the increase was in fact comparable in both seasons, with sulpiride inducing a 2.2 fold increase in secretion during the summer and a 2.7 fold increase during the winter. This suggests that individual lactotrophs have much the same capacity to respond to dopamine receptor blockade in the summer and winter periods i.e. individual cells can double their prolactin output upon removal of tonic dopaminergic inhibition. It is possible that the slightly greater increase following sulpiride treatment of winter fetuses reflects the lower basal secretion of prolactin from the active lactotrophs during the winter thereby allowing a greater store for release following removal of the inhibitory influence of dopamine.

The significantly reduced number of functional lactotrophs in the winter pituitary and the finding that prolactin concentrations in winter fetuses are lower than in summer fetuses, even after the removal of the inhibitory dopaminergic tone may reflect the suppressive influence of melatonin. Maternal pinealectomy abolishes the diurnal rhythm of melatonin in both the ewe and fetus (Yellon and Longo, 1988; McMillen and Nowak, 1989) whilst infusion of melatonin to pregnant ewes during long days, to mimic the short day duration of the melatonin signal, results in decreased prolactin concentrations in both the maternal and fetal plasma (Bassett *et al.*, 1989). Therefore, the duration of the nocturnal melatonin release in the pregnant ewe and thus, in the fetus is increased during the winter and this in turn decreases plasma prolactin concentrations in both the ewe and the fetus. The mechanisms by which melatonin acts to regulate the secretion of pituitary prolactin are not fully understood. It seems likely that melatonin exerts its influence directly at the level of

the pituitary since disconnection of the pituitary from the hypothalamus does not disrupt the photoperiodic regulation of prolactin secretion (Lincoln and Clarke, 1994). More recently, hypothalamic-pituitary disconnection of the fetus has confirmed that the fetus also maintains the prolactin response to external photoperiod in the absence of a functional link between the hypothalamus and the pituitary gland (Houghton, Young and McMillen, 1995). The persistence of a prolactin response to external photoperiod in HPD adults and fetuses suggests that melatonin has an extrahypothalamic mode of action, that is, melatonin acts directly at the level of the pituitary gland.

In the present study, the approximately two-fold increase in plasma prolactin concentrations in both photoperiods following sulpiride infusion suggests that the level of tonic dopaminergic inhibition on pituitary lactotrophs is similar in summer and winter fetuses. The twofold increase in prolactin concentrations in both photoperiods is in accordance with the data presented by Houghton *et al.* (1995). In their study of HPD fetuses maintained under short and long days, Houghton *et al.* administered a bolus of the dopamine antagonist chlorpromazine intravenously at day 141-142 gestation. They reported that the increase in plasma prolactin in intact fetuses in response to chlorpromazine was the same in both photoperiods when expressed as a percentage increase from baseline values. HPD fetuses did not respond to chlorpromazine in either photoperiod indicating that the effects of external photoperiod on fetal prolactin secretion are not mediated entirely by the inhibitory influence of hypothalamic dopamine on pituitary function.

In this respect, Lincoln and Tortonese (1995) investigated the extent to which dopaminergic pathways are involved in the prolactin response to chronic administration of melatonin in the adult sheep. Sheep were treated with micro-implants of bromocriptine or sulpiride given alone or in combination with melatonin. Bromocriptine given in combination with melatonin produced the same effect as melatonin give alone; a significant decrease in plasma prolactin concentrations. Sulpiride given in combination with melatonin also produced the same effects as melatonin alone. The authors concluded that it is unlikely that the short day melatonin signal acts through a hypothalamic dopaminergic system to inhibit the secretion of prolactin since administration of bromocriptine in combination with melatonin did not further suppress prolactin concentrations when compared to the effects of melatonin alone and sulpiride given in combination with

melatonin was unable to negate the effects of melatonin. The hypothesis that melatonin acts to suppress plasma prolactin concentrations independently of dopamine is supported by the finding that sulpiride treatment of HPD rams under short days or after melatonin treatment failed to elicit the release of prolactin (Lincoln and Clarke, 1995).

In the present study, since it was not possible to construct dose-response relationships, it is not possible to determine the extent to which the level of dopamine receptor expression in winter and summer pituitaries is involved in mediating the response to bromocriptine and sulpiride. However, it is interesting to speculate that the attenuated response to bromocriptine in summer fetuses reflects a change in the level or specificity of dopamine receptor expression. It is possible that the overriding inhibitory presence of dopamine during the winter months acts to desensitise the receptors whereas the reduced dopamine influence in the summer has the opposite effect. This would mean the effect of a given dose of dopamine agonist would be more effective in the summer when the receptors are more sensitive. Alternatively, the increased response to bromocriptine in the summer could simply reflect the increased number of lactotrophs present and therefore, presumably, an increased level of dopamine receptor expression. However, these explanations appear to be at odds with the similar response of both summer and winter pituitaries to dopamine receptor blockade, which suggests that the number of receptors and their level of sensitivity is comparable in both photoperiods. Studies investigating the dose-response to bromocriptine and sulpiride in both seasons are needed to clarify this point.

In conclusion, the study presented in this chapter demonstrates that the secretion of prolactin into the fetal circulation is subject to tonic dopaminergic inhibition in both the summer and winter months and that dopaminergic inhibition is not solely responsible for low plasma prolactin levels during the winter months .

Chapter 10. General Discussion

The sequential maturation of the fetal hypothalamo-pituitary-adrenal axis is essential for the co-ordinated development of many organ systems and in species such as the sheep is pivotal in the initiation of parturition. The studies described in this thesis aimed to investigate the neuroendocrine regulation of hypothalamo-pituitary function during fetal life.

The timely maturation of the hypothalamic neuropeptides CRH and AVP is essential for the subsequent induction of ACTH release from the fetal pituitary gland. However, little is known about the factors controlling the development of these important hypothalamic neuronal populations. The first objective of the studies presented in this thesis was to chart the development of hypothalamic AVP neurons in cell culture in an attempt to investigate the role of putative neurotrophic factors on the growth and functional maturation of this neuronal population. The study presented in chapter 4 described the maintenance of fetal rat hypothalamic neurons in cell culture for periods of up to 20 days. The functional maturation of AVP neurons in vitro was not significantly altered by treatment with IGF-1 however, due to the inability to specifically identify AVP neurons within the culture system it was not possible to elucidate any possible effects of IGF-1 on the rate of neurite outgrowth of the AVP neurons. Whilst cell culture is an invaluable tool in the analysis of complex systems such as the hypothalamo-pituitary axis, the study presented in Chapter 4 illustrated limitations of such a technique. Thus it was not possible to determine whether the inability to identify AVP neurons in cell culture reflects the insensitivity of the immunocytochemistry procedure or a fundamental difference between AVP neurons in vivo and in vitro. At present, the identity of factors regulating the growth and development of AVP neurons in the fetal hypothalamus remains undetermined. This is a most interesting area and one which clearly warrants further study. However, it seems that an alternative approach is necessary before specific effects of putative growth factors, such as those on neurite extension and differentiated morphology can be investigated. One such approach might be the use of explant cultures. One can envisage extension of neurites from fetal hypothalamic explants towards the source of a putative growth factor. Fixation and processing of the entire explant may overcome some of the problems of cell culture experienced in the study of Chapter 4.

The remainder of the studies presented in this thesis dealt with the ontogeny and dopaminergic regulation of the fetal pituitary gland. Chapters 6-8 revealed that secretion of the POMC-derived peptides α-MSH and ACTH was subject to tonic dopaminergic inhibition. Thus, bromocriptine treatment significantly reduced and sulpiride treatment significantly increased the concentrations of α-MSH in the fetal circulation. In addition, circulating ACTH concentrations were significantly increased following sulpiride treatment. There was no influence of either treatment on pituitary α-MSH or ACTH content and, whilst bromocriptine treatment resulted in a significant reduction in the level of POMC mRNA present in the intermediate lobe there was no effect of sulpiride. These results suggest that the dopaminergic regulation of POMC-derived peptide secretion from the fetal sheep intermediate pituitary is complex (see Figure 10.1).

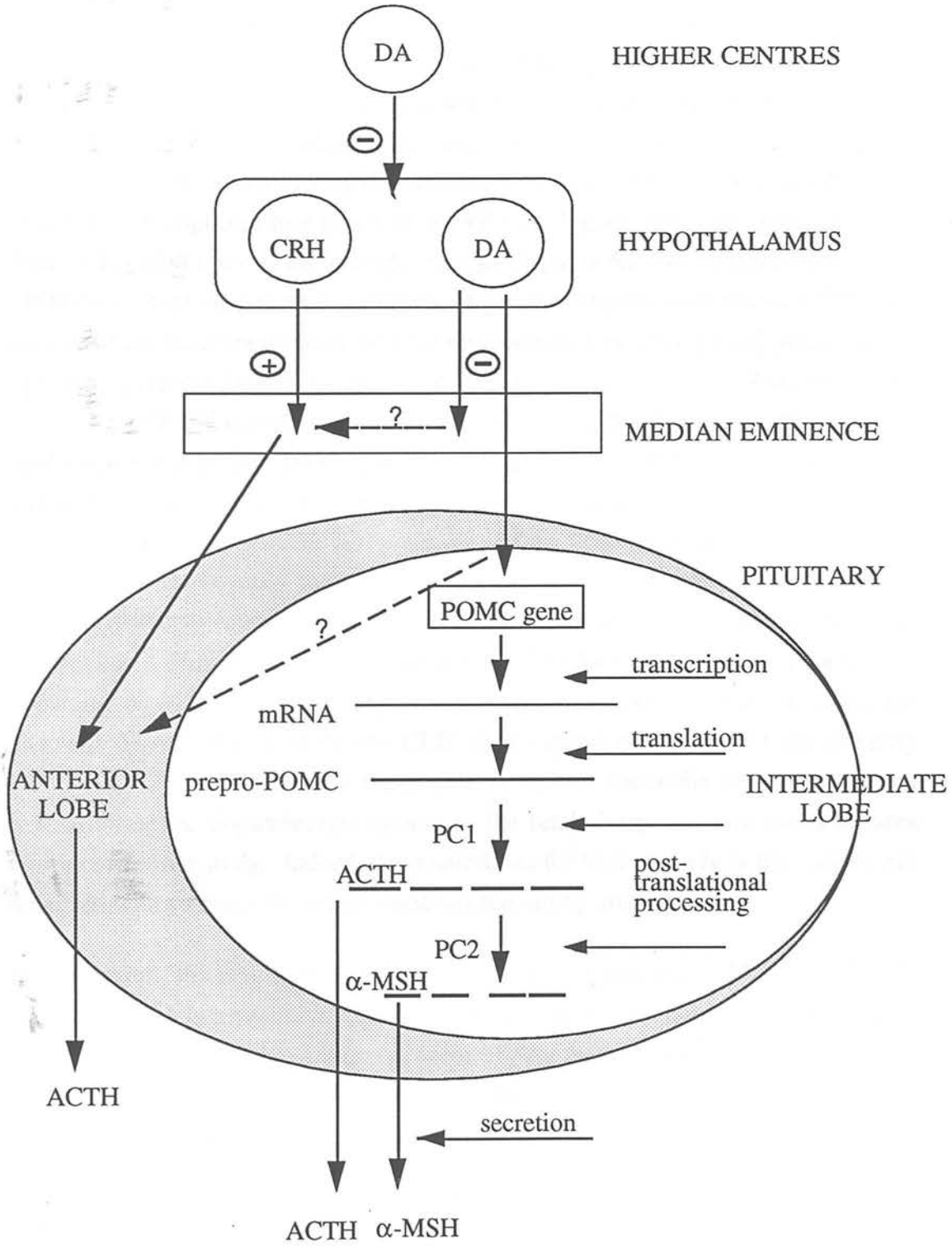
In the adult rat, long-term administration of dopamine agonists induces a decrease in the content and secretion of POMC-derived peptides and reduce the rate of POMC biosynthesis by decreasing POMC mRNA levels in the intermediate lobe (Beaulieu *et al.*, 1984; Chen *et al.*, 1983; Millington *et al.*, 1986). Conversly, dopamine antagonists produce a sustained increase in the content and secretion of POMC-derived peptides and accelerate the rate of POMC biosynthesis by elevating intermediate lobe POMC mRNA expression (Chronwall *et al.*, 1987; Hollt *et al.*, 1982, Millington *et al.*, 1986). However, the results presented in this thesis suggest that this is not the case in the fetal sheep. Whilst the decrease in circulating α-MSH concentrations in response to bromocriptine can be directly attributed to a decline in POMC mRNA levels, the situation in response to dopamine receptor blockade by sulpiride is more complex. Plasma α-MSH concentrations are rapidly increased following sulpiride treatment. As discussed in Chapter 6, this response is indicative of a readily releasable pool of α-MSH from the fetal intermediate lobe. However, plasma α-MSH concentrations remain high for the duration of the 72 hour treatment period without depletion of intra-pituitary α-MSH content (Chapter 7) or any alteration in the level of POMC mRNA expression (Chapter 8). Therefore, hypersecretion of α-MSH induced by removal of inhibitory dopaminergic tone must reflect altered processing of the POMC molecule post-transcriptionally. Post-transcriptional regulation of POMC mRNA may occur by attenuation of the rate of translation to produce the POMC protein precursor, an increased rate of post-translational processing of the precursor molecule or a combination of both events. These events, either alone or in combination would account for the pattern of gene

expression, peptide secretion and pituitary content described in this thesis in response to sulpiride. Thus, the fetal pituitary is capable of inducing a complex array of regulatory mechanisms in order to supply the increased demand for peptide. Dopamine could regulate these events at any number of sites in the POMC pathway. Potential sites for dopaminergic modulation are illustrated in figure 10.1. The results presented in this thesis suggest that post-transcriptional events are involved in the acute regulation of POMC-peptide expression by dopamine however, it is likely that long-term regulation (ie. longer than 72 hours) involves modulation of the rate of gene transcription. The mechanisms whereby dopamine modulates these post-transcriptional events remains to be elucidated. Other potential sites of actions of dopamine may include action on CRH nerve terminals in the median eminence and possible interaction between dopamine released at the intermediate lobe with the anterior lobe of the pituitary gland (shown as question marks in Figure 10.1).

The studies presented in this thesis represent investigation of only one possible regulatory mechanism influencing intermediate lobe function during fetal life. It is likely that other neural factors are involved in control of intermediate lobe function during development. Evidence for this comes from the finding that treatment with bromocriptine alone fails to fully restore pituitary function after hypothalamo-pituitary disconnection. In addition, whilst haloperidol tended to increase intermediate lobe α-MSH and α-N-acetylated endorphin content (Smith *et al.*, 1989; as discussed in Chapter 7), the increase was substantially less than that induced by HPD. Another neural factor likely to be involved in regulation of intermediate lobe function is γ-amino butyric acid (GABA). GABA-ergic projections to the intermediate lobe have been identified in the adult rat (Vincent, Hokfelt and Wu, 1982) and GABA-mediated inhibition of α-MSH release has been demonstrated (Tomiko, Taraskevich and Douglas, 1983). Therefore, it is important to appreciate that no one system is likely to govern the regulation of the many-POMC-derived peptides from the fetal pituitary gland but rather many systems act in concert to maintain a balance of peptide production and secretion.

One important finding of this thesis is that, in direct contrast to previous reports (Llanos *et al.*, 1979; Glickman *et al.*, 1979; Baird *et al.*, 1983) α-MSH does not appear to have any steroidogenic effects on the fetal adrenal gland in late gestation. This raises the original question as to the role of the intermediate lobe and α-MSH

Figure 10.1. Schematic diagram illustrating potential sites of dopaminergic regulation of the POMC pathway in the fetal sheep pituitary gland. Results presented in this thesis suggest that post-transcriptional events are important in the initial response to dopamine blockade. Long-term regulation of POMC-derived peptide expression is likely to involve regulation at the level of gene transcription (solid arrows). Additional dopaminergic influence on POMC products may occur in the anterior lobe of the pituitary via inhibitory effects on hypothalamic CRH nerve terminals in the median eminence or interaction between dopamine released at the intermediate lobe with the anterior lobe of the pituitary gland (dashed arrows).


DA
HIGHER CENTRES
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PITUITARY
POMC gene
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transcription
mRNA
translation
ANTERIOR LOBE
prepro-POMC
INTERMEDIATE LOBE
PC1
ACTH
post-translational processing
PC2
α-MSH
ACTH
secretion
ACTH
α-MSH

during fetal life. It is clear from the ontogeny study presented in Chapter 5 that the fetal pituitary abundantly expresses α-MSH at a very early stage during fetal development. Investigation into the nature of this peptide in the early stages of development is necessary to determine whether it is actively secreted into the fetal circulation and if so is it active at the fetal adrenal gland during the early stages of development. As it stands, very little is known about the role of α-MSH in the fetus. Whilst it is recognised that levels of α-MSH are higher in the late gestation fetus than in the adult there is no information regarding the relative concentrations of α-MSH throughout the course of gestation. A possible hypothesis is that α-MSH is the peptide of choice released from the fetal intermediate lobe during early gestation and that it is preferentially replaced later in gestation by ACTH. This would be inkeeping with the reported influence of α-MSH on growth and maturation of the fetal brain and placental development (Swaab and Martin, 1981). Regulation of this nature could occur by temporal changes in the expression of prohormone convertase enzymes. Thus, it is possible that processing of the POMC precursor molecule early in development favours the production of α-MSH and that the rate of both processing and secretion of α-MSH is reduced following the onset of dopaminergic innervation. This would be consistent with the observed negative influence of dopamine on PC2 expression, the prohormone convertase enzyme that facilitates the cleavage of ACTH to a-MSH and CLIP in the intermediate lobe of the pituitary gland (Day *et al.*, 1992). To date, little is known about the development and regulation of the dopaminergic system in the fetal sheep and this too is an area worthy of further study. Indeed, the possibilities for further study in this system are manifold with so many important questions remaining unanswered.

In conclusion, the results presented in this thesis suggest that α-MSH and ACTH from the fetal intermediate lobe are not fundamentally important for the late gestation drive in adrenal stimulation necessary for the initiation of parturition in the sheep. The extent to which POMC-derived peptides other than ACTH are involved in this process remains to be determined. However, the result presented in this thesis do not preclude a role for α-MSH in the development and maturation of the fetal adrenal gland nor do they rule out a potential role for other POMC-derived peptides of the intermediate lobe, notably the N-terminal fragments in the late gestation increase in adrenal function.

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